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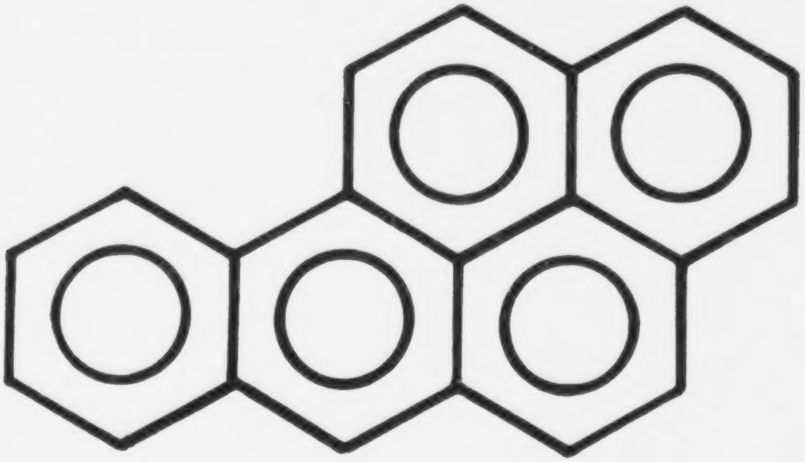

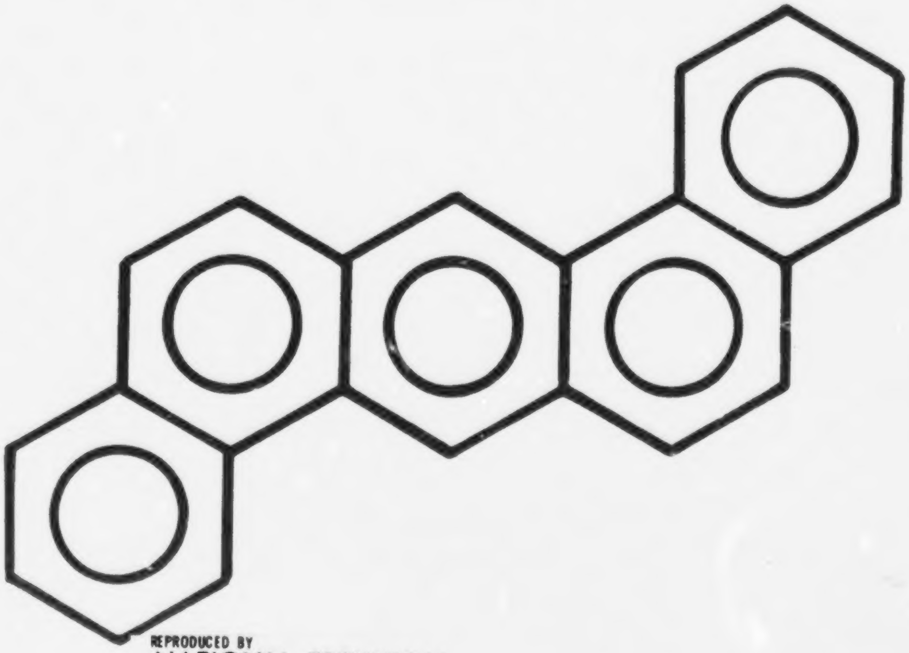

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SYMPOSIUM:

CARCINOGENIC POLYNUCLEAR AROMATIC HYDROCARBONS IN THE MARINE ENVIRONMENT

Pensacola Beach, Florida
14-18 August 1978

Edited by
N.L. Richards and B.L. Jackson
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ENVIRONMENTAL RESEARCH LABORATORY
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U.S. ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants required that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze (ERL,GB), contributes to this information through research programs aimed at determining:

> the effects of toxic organic pollutants on individual species and communities of organisms;
>
> the effects of toxic organics on ecosystem processes and components;
>
> the significance of chemical carcinogens in the estuarine and marine environment.

This publication is a compilation of papers contributed by scientists who participated in the Symposium on "Carcinogenic Polynuclear Aromatic Hydrocarbons in the Marine Environment" sponsored by ERL, Gulf Breeze and the Environmental Protection Agency (EPA) Office of Energy, Minerals, and Industry August 14-18, 1978, at Pensacola Beach. Participants addressed a number of questions related to the impact of these compounds on the marine ecosystem: their physical, chemical, and biological fate after release into the aquatic environment; the current state-of-the-art for their detection and identification; and their potential for transfer to human food.

Henry F. Enos

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OVERVIEW

by

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Development of an EPA research program to assess the potential for mutagenic or carcinogenic polynuclear aromatic hydrocarbons (PAHs) to reach man through the marine food web was a formidable undertaking. An understanding of the complex marine sources and sinks of PAHs and the processes involved in the evaporation, dissolution, biological uptake, and transformation at different trophic levels of food webs is only beginning to emerge in the literature. Our effort required a multidisplinary team approach to a rapidly expanding field of environmental science.

This symposium was convened primarily to summarize current knowledge about the potential for carcinogenic PAHs and their metabolites to accumulate in seafood organisms. Although speakers presented papers in highly specialized areas, the interrelationship of their research will be obvious to readers. For example, chemists with expertise in carcinogen/mutagen separation and identification from complex environmental mixtures can now team up with biologists who have sensitive biological carcinogen/mutagen detection methods and study a wide variety of problems. Another breakthrough involves a better understanding of the induction of PAH metabolizing enzymes in marine animals as a possible monitoring tool.

Topics for invited papers ranged from the development of sensitive new techniques for analytical chemical detection and fate of PAHs to their detection through the use of biological indicators. Recent developments in metabolism by marine organisms, effects of genotype on responses, and the cellular fate of PAHs these compounds were discussed. Speakers included internationally known authorities in such disciplines as analytical chemistry, photochemistry, enzymology, physiology, molecular biology, genetics, aquatic toxicology, microbiology, and fishery biology.

Research results supported the belief that additional breakthroughs will be forthcoming that will improve our understanding of the potential for PAHs to reach man through the marine environment. Interspecies comparisons illustrate both similarities and differences in PAH uptake, metabolism and detoxification. Biological methods with potential for improved detection of mutagens/carcinogens in complex environmental mixtures were described. An understanding of the role of heredity, exposure route, immunology, metabolism, and detoxification mechanisms in a diverse range of marine organisms was recognized as essential to predict the outcome of exposure of marine organisms to PAHs.

The release of PAHs into the marine environment is likely to increase in response to increased petroleum transportation and offshore oil exploration and production. The possibility also exists that in the distant future a shift to fuels derived from shale oil may expose the marine environment to PAHs of different chemical composition. Because the photochemistry of PAHs is now better understood, we may acquire new insight into the weathering of PAHs in the marine environment and thus into the selection of appropriate model compounds for toxicity and bioaccumulation studies.

We now have many of the research tools required to assess the potential for mutagenic/carcinogenic PAHs and their degradation products to accumulate in seafood organisms. An intensified research and monitoring program would help resolve questions regarding the human health risk when mutagenic/carcinogenic PAHs enter the marine environment.

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REVIEW OF SPECIES-SPECIFIC METABOLIC PATHWAYS OF CARCINOGENIC POLYNUCLEAR AROMATIC HYDROCARBONS IN MARINE ORGANISMS*

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ABSTRACT

The problem of neoplastic growth is a fundamental one to man and to organisms in the surrounding environment as our environment becomes more and more polluted. The marine environment, the largest on earth, has great import for the future, affecting aspects of nutrition, transportation, resource recovery, energy, and recreation.

We know from Aristotle, Organon, Part IV, that we can attack a problem by its general feature, known in philosophy as katholou, in contrast to kathekaston, which is the specific. It is a pleasure to speak in this symposium on the general comparative aspects of the metabolic pathways of carcinogenic polynuclear aromatic hydrocarbons in marine and other organisms. Other presentations will deal with specific topics of these compounds in marine organisms. It is necessary for our review to include current knowledge of nonmarine organisms, including man and nonmarine plants, to trace briefly the action of carcinogenic polynuclear aromatic hydrocarbons and their species-specific metabolic pathways. Finally, we shall point to the necessity for stimulating new research for protecting the marine environment. The polycyclic aromatic hydrocarbons, a most important group of chemical carcinogens, are the concern of this symposium. Improvements in the methods for the detection

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* Shortened version of address

of the carcinogenic polynuclear aromatic hydrocarbons and other compounds have facilitated studies concerning their fate in the oceans, as well as comparative evaluations of the species-specific, organ-specific, tissue-specific, and pathway-specific processes leading to carcinogenesis. The recent successes in fusing cell structures of animals (including man) and plants give new impetus to a broad comparison of species-specific metabolic pathways of the carcinogenic polynuclear aromatic hydrocarbons in the organisms of the marine environment. The pathways of chemical compounds can be compared with respect to the following topics: contact of the carcinogen(s) with tissue(s), absorption, storage, effect of circadian and other rhythms, and metabolic interactions. These may include species-specific differences in enzyme systems involved in the metabolism of carcinogens, differences in the chemical binding of carcinogens or their metabolites to cell constituents, differences in biochemical constituents in the cell during carcinogenesis, variable pathways of the same carcinogen, the excretion of carcinogens, and the influence of the altered metabolism of the neoplastic tissues on the total organism. These changes express themselves simultaneously in morphologies of histogenesis and in metabolism.

A full understanding of the broad range of the topic requires a consideration of the framework of comparative pathology in general. Unfortunately, the information available on metabolic and other pathways of carcinogenic polynuclear aromatic hydrocarbons in marine organisms is still rather scarce. Historically, basic discoveries in this area have involved primarily nonmarine organisms.

INTRODUCTION

Abnormal growth is a complex but fundamental problem of life. Neoplastic growth is the most pervasive type of abnormal growth for man and the surrounding environment. (The marine environment of concern to this symposium is important for the future, affecting aspects of nutrition, energy, resource recovery, transportation, and recreation.) The ocean as the source of nutrition for the future concerns us indirectly with regard to the life change of many inhabitant groups that serve as food for fishes and for marine mammals, such as whales, sea cows, etc.; marine flora also may undergo deleterious effects. These developments have serious economic significance for man. Directly, we are concerned with the chain reactions of toxicants in the ocean because of the possible contamination of food that enters our own bodies. Many years ago it was noted that molluscs are able to store carcinogenic hydrocarbons, for example, benzo(a)pyrene (Cahnmann and Kuratsune, 1957). (Later we shall see differences in the reaction of molluscs and mammalian integument to hydrocarbons.) Certain organisms may only store hydrocarbons, others may metabolize them toxicologically with higher carcinogenic power than the parent substance, and others may simply detoxify them. Thus we need to observe species-specific interaction and differences in this regard, as well as species reactions with nonliving matter.

Regarding energy, we may distinguish between clean energy as that produced from the tides, the waves, and different temperatures in varying layers of the waters of the oceans and dirty energy, which refers to resource recovery as it concerns deep-sea and shelf mining. The primary sources of energy in all organisms come from the sun by organismic conversion of sun rays and inorganic matter through the process of photosynthesis, making the plants the basic elements of all life functions.

It is impossible to understand pathological processes without a preliminary understanding of normal processes. Comparative pathology is based on three pillars: namely, comparative histology, comparative embryology, and, of special concern to us, the normal distribution of chemical compounds in the organisms.

SPECIES SPECIFICITY OF METABOLISM

In considering species specificity of metabolism, we shall see that diseases can be seen from three aspects: (1) the organisms they attack, (2) the causative agents or types of a disease, and (3) the life functions impaired by a disease. Neoplasms, the most important diseases caused by carcinogenic hydrocarbons that may also be mutagenic, are common (at least theoretically) to all organisms with true tissues. If we look at the comparability across taxonomic borders, we can state that the compounds of interest to us in this symposium are able to produce neoplasms in a large number of organisms in animals and plants alike. It is of interest to note that we can compare morphological structures of animals to those of plants if, for example, we equate the sex organs of the human female to such a spring flower as the snow drop, more specifically in this regard, the uterus in the human to the ovary in the plant. The glands in plants as well as in animals are composed of secretory and supporting cells. Tissues of both kingdoms in the whole sense are comparable, especially as concerns the lining membranes, but there is also incomparability of certain tissue groups. It has been well-known for many years that animal and plant cells show certain differences but can be compared easily in general characteristics. [It is of special importance to our understanding of the organisms tissues we deal with today and their biochemical pathways in the cell that it was possible for Jones and coworkers (1976) to implant plant plastids into human Hela cells and that Lima-de-Faria (1981) was similarly able to fuse human and plant cells or their parts.]

Carcinogenesis, and Chemical Carciogenesis in Particular, a Multistep Process with or without Species-specific, Organ-specific, Tissue-specific Variation

Berenblum (1974) stated: "A clue to the biochemical mechanism of carcinogenic action can sometimes be derived from indirect evidence, e.g., by studying differences in species and organ response to carcinogenic action when these are associated with distinctive patterns of metabolic changes in the causative agents."

This statement explains the intention to view comparative pathology on as wide a background as possible. We can state as a rule "the wider scientific valuable frame of comparative pathology, the deeper the understanding we gain." Today the attention of pathology has been shifted from a science dealing with diseased organs to a science dealing with biochemical, biophysical, and ultrastructural changes in diseased cells. But there are not only the primary factors, such as a particular carcinogen and cell constituents shaping the process of carcinogenesis, but also many factors, until now little known, which may be called secondary factors. In experiments undertaken several years ago it could be shown that, for example, a carcinogenic solution of 1% benzo(a)pyrene in an organic solvent was fast expelled from different species of coelenterates, such as *Tealia felina*, *Sargatiogeton* sp., *Actina equina*, and *Metridium senile*, and led to an immediate secretion of the compound into other invertebrates, such as the pulmonate, *Arion subfuscus*. It resulted in the building of resins and simply a foreign body reaction. In the case of implantation of the compounds, toxic results only could be attained in different species of starfish; however, granuloma production was observed after two months of a one-time implantation in the starfish, *Solaster papposus*. It is our opinion that temperature in the different body fluids and the solubility of the carcinogen and different types of body fluids and circulatory systems may play a more or less important role leading to direct neoplastic transformation, in addition to the primary factors in the interaction of initiators, promoters, and the cell (Kaiser, 1965).

In the comparison of plants and animals, we should remember that the two sources where most spontaneous neoplasms and growth anomalies have been observed are in man himself and in the angiosperms. There are over 14,000 growth abnormalities, known as galls, limited and unlimited in the growth potential, described in plants. There are tissues in plants, such as the meristems, not present in the vertebrate. These are the tissues with at least theoretically unlimited growth potential during the life span of a vascular plant. There are no muscular or nervous tissues in plants. To go back once more to the meristematic tissues, we must state that the most comparable tissue structures among animals occur in cirriped crustaceans (larval tissues) or imaginal discs of such creatures as the seventeen-year locust. Invertebrates also exhibit such developmental round-abouts as placentae. Further, we know that embryonal development and tumor development can run in parallel ways. The role of ontogenetic transformations in larval developments, including normal histolysis and biochemical processes and their changes during embryonal development, is a widely neglected field of oncology, particularly in the area of biochemical pathways of carcinogens. Marine organisms with placenta development are *Salpa primata* and *Thalia democratica* of the class Thaliacea, phylum Tunicata.

Contact of carcinogen with tissue--

Epithelium in animals and lining membranes in the plants are the tissues in the organisms susceptible to contact with hydrocarbons, which act in contrast to the aromatic amines, at the site of application.

The integuments and body coverings permit a broad comparison of how contact occurs between the carcinogens and the organism investigated and affected by the compound(s). Special emphasis must be put on the distribution of the different tissue types which make certain variations in this regard. Special emphasis has to be put on excretion of the tissues, such as mucous layers, cuticles and exoskeletons, on one hand, and surface specialization, such as villi and cilia, on the other. A direct effect of the carcinogenic process on these structures can be distinguished from an indirect one. A direct effect may play a role in the process or prevention of carcinogenesis (Arcadi, 1977): 7,12-dimethylbenz(a)anthracene, a potent carcinogen of papillary tumors in mice, did not produce skin tumors in the mollusc, *Lehmannia poireri*. Such different effects may have something to do with the mucous layer of the gastropod. We used the gill epithelium of the bivalves, *Unio* or *Mytilus*, which are ciliated, to check ciliastatic components in cigarette smoke condensate. The phenolic compounds have been found to be not only promoters in carcinogenesis but also potent ciliastatic compounds. This side effect can be seen as an indirect influence of an otherwise cocarcinogenic compound. The comparison is possible because nearly all cilia in both kingdoms of the two-kingdom approach are built similarly (Wynder *et al*., 1963).

Absorption--

In the second step, absorption, important general differences are exhibited by animals and plants. Animals, with few exceptions, lack nonliving tissues. However, plants have nonliving tissues, as well as rigid cell walls, whereas the animal cell can be considered nude. Cuticles occur in both groups, for example, the exoskeletons of crustaceans or the wax cuticle on the leaf of red cabbage. The typical excreted cell wall of the plant is composed of cellulose; in the case of fungi (especially the higher ones), of chitin. Some cells of flagellates, lower fungi, and sex cells are also nude in plants (in the two-kingdom approach). This is of little importance because by definition such lower organisms exhibit no true tissues necessary for neoplastic development. There is a significant difference between animals and plants in that the plant needs a wound in most cases for absorption of chemical carcinogens for reasons mentioned previously. This is true for autonomous plant neoplasms, such as crown gall disease, the wound tumor virus disease, or galls and neoplasms produced by chemicals. The only exceptions are the genetic tumors in plants. In addition to the species-specific differences of absorption, regional differences exist also, for example, in the skin of mice. Further complications are occasioned by such factors as circadian and other rhythms.

The surface area of different species of plants and animals was treated with a 1% solution of benzo(a)pyrene in acetone or DMS. The treated skin area was removed at various time intervals. The surface area was then exposed to 2 mℓ of acetone and injected in the 881 Perkin Elmer

Gas Chromatograph and the Cary Model 15 Spectrophotometer. The absorption rate of carcinogenic polycyclic hydrocarbons varies: (1) in animals a stepwise absorption occurs, whereas in plants the main absorption takes place at once; (2) differences in absorption do not occur in areas where the strongest carcinogenic effect is produced. These facts suggest that the fastest rate of absorption in plants, as in animals, does not necessarily accompany the strongest carcinogenic effect. The metabolic interaction of the carcinogen with the treated species at specific time intervals appears to play a more important role (Theisz et al., 1966).

Storing in tissue--

In the storing of chemical compounds in tissues, the time element becomes a significant factor. The metabolic interaction between a chemical compound and cell organelle of a particular species requires a specific time elapse. For example, it is very hard to produce neoplasms with chemical carcinogens in the frog, because the frog has a large lymphatic system that eliminates carcinogens very rapidly (because of enzyme influence, etc.). Diffusion rates can be species-specific in the same site of injection, as shown with the carcinogenic hydrocarbon 7,12-dimethylbenz(a)anthracene. It remained for a long time in the subcutis of mice and rats where it was carcinogenic, but was eliminated very fast in rabbits (Berenblum, 1954).

Equally important is the time at which a carcinogen or a chemotherapeuticum is applied to a particular species. The activity, amount, and presence of such constituents as enzymes vary, thus playing a role in the interaction, enabling the formation of pathways of the carcinogens, and determining which compound may be actively carcinogenic in one species and not in another, or only during a particular time interval (Edmunds and Halberg, 1981).

Time Factor of Application--

The biochemical reactions of different species can be affected by chronobiological influences. This holds true in that the various types of rhythmicity of species differ because of variations in life span, life habits, food supply, environment, and other aspects. It need only be realized that the mouse, for example, is a nocturnal animal, whereas man is a day creature. However, direct comparison from chronobiology alone is impossible if rhythmic differences are not included in the calculation.

Duration of Stay--

In general the period of chemical carcinogenesis can be divided thus: the time interval during which the neoplastic transformation occurs and a second phase, the progression from the first cancer cells to the tumor development. Concerning the organismic background of our environment, the chain reaction of different organisms and the interaction of morphological and biochemical/biophysical aspects may be distinguished. The chain reactions between different organisms can be positive or negative regarding aflatoxin (Ferrando et al., 1977): Diet including 20 p. 100 of lyophilized milk produced by a goat that consumed peanut meals containing 1530, 79, and

54 µg/kg aflatoxin with 1136, 64, and 54 µg/kg aflatoxin B1 was fed to duckling for 23 days. Direct consumption of peanut meals, with the highest level of aflatoxins, produced 18 p. 100 mortality and characteristic injuries of aflatoxicosis.

Fischer and Horvath (1977) found that microvilli of *Tubifex tubifex* Müll. of the supporting cells of the epidermis play a significant role in repairing cuticular injuries. Acid mucopolysaccharides of the cuticle and epidermis may function as traps for heavy metals, as proven by their significantly heavy metal content. The cytosols of the epidermal cells possess considerable DAB-reactivity. The enzyme, responsible for the DAB-reaction, may be transported by the microvilli toward the cuticular surface and can play a central role in the detoxication of organic foreign compounds.

Species-specifc differences in enzyme systems involved in the metabolism of carcinogens--The different possibilities of metabolic pathways of chemical carcinogens exhibit more or less pronounced species specificity. Weisburger and Weisburger (1958) found that the urinary excretion of the various ring hydroxy metabolites of ^{14}C-labelled AAF in the highly responsive rat differed greatly from the nonresponsive guinea pig. The 7-OH metabolite dominated in the guinea pig and while the levels of the 1-, 3-, 5-, and 8-OH metabolites were greater in the rat. Hydroxylases convert the polycyclic aromatic hydrocarbons into phenolic derivatives in the microsome and endoplasmic reticulum of liver cells. A few biochemical characteristics of hydroxylation of different aromatic hydrocarbons have been demonstrated. Methylcholanthrene, administered by daily intraperitoneal injection to rat liver (40 mg/kg body weight), resulted in elevated hepatic levels not only of malic enzymes but also of the pentose phosphate pathway dehydrogenases (Shas and Pearson, 1978). A few metabolic mechanisms of the biotransformation by direct oxidative alkyl chain cleavage in the vicinity of the hydroxy group of (2-hydroxybutyl)-N-butylnitrosamine were demonstrated by Blattman (1977). Acetaminophen is metabolized through a variety of pathways, as shown by Thorgeirsson and coworkers (1978). The prevention of benzo(a)pyrene-induced mutagenicity by homogeneous epoxide hydratase was described by Oesch and coworkers (1976). Differences in enzyme content and chronobiological rhythms also seem to play a role in racial variations of breast cancer in women of different countries as shown by Halberg (1981).

Species-specific differences in chemical binding of carcinogens or their metabolites with cell constituents--Carcinogens themselves or their metabolites seem to act by a chemical mutation in DNA genes of the nucleus. Differences exist among species regarding the binding capacity of chemical carcinogens and also of the binding site with regard to varying carcinogens. Variations also occur concerning the binding of carcinogenic and noncarcinogenic compounds of the same chemical type.

It is not always compelling that the largest amount of a carcinogen in a particular organ may cause the largest amount of neoplasms. 1,2-dimethyl hydrazine administered to rats showed that 96% brought with bile to the intestine was less effective than the 4% received by the intestinal wall through circulation. The small amount of the compound metabolites played a leading role in the genesis of intestinal tumor according to Pozharisski and coworkers (1976). Comparable to two separate components in a methanol-sodium borate solution gradient, as in the case of benzo(a)pyrene, 2-stereoisomeric diolepoxides are involved in the binding of 7,12-dimethylbenz(a)anthracene to DNA in culture cells. The binding of the carcinogen does not decrease the overall metabolism of the carcinogen in a remarkable way. Comparative studies of Roebuck and coworkers (1978) demonstrated that no specific pattern of toxicity or carcinogenicity was connected with the liver metabolism of aflatoxin by the duck, rat, mouse, monkey, and humans.

Species-specific differences in biochemical changes in the cell durings carcinogenesis--According to the large number of organisms with true tissue, the species-specific variations of the action cell components with carcinogens are very limited, especially for organisms of the marine environment. We know absolutely nothing about the biochemical cellular changes of most of the minor phyla, but it would be easy to do more experimental work and gain interesting new results with these organisms and their physiology. It would be necessary only to apply to these organisms the methods used for terrestrial animals, such as the mouse and rat, or aquatic animals, such as fish.

A few years ago, Harshbarger and coworkers (1971) investigated the effects of carcinogen-contaminated water on crustaceans. More recently, Khudoley (1977) studied the effect of diethyl- and dimethylnitrosamines dissolved in tank water (200-400 ppm), which induced basophilic cell neoplasms in 16 of 95 and in 6 of 17 molluscs of the species, *Unio pictorum*. Neoplasms occurred in the hepatopancreas. Methylnitrosoguanidine produced only inflammatory reactions at the injection site. It is suggested that these invertebrates be used as indicators of hydrospheric pollution with chemical carcinogens. A later paper reported the cellular transformations of polycyclic aromatic hydrocarbons. The interaction of N-acetoxy-N-2-acetyl- aminofluorene and its binding to the regions of chromatin sensitive to enzymes in duck erythrocytes were reported by Metzger and coworkers (1976); tumor growth in molluscs was reviewed by Khudoley and coworkers (1977).

Variable pathways of same carcinogen in the same tissue of the same organism--Similar to normal chemical compounds, carcinogens are also metabolized in different ways, sometimes in the same species. Two cytochromes, for example, from liver microsomes of the rabbit treated with 2,3,7,8-tetrachlorodibenzo(p)dioxin appear to be distinct entities and function in different catalytic pathways (Johnson and Muller-Eberhard, 1977).

The main urinary metabolite of safrole in the rat and man was excreted in a conjugated form. Small amounts of eugenol or its isomer 1-methoxy-2-hydroxysafrole, approximate carcinogen of safrole, and 3'-hydroxyisosafrole were detected as conjugates in the urine of the rat. However, Benedetti and coworkers (1977) were unable to demonstrate the presence of the latter metabolites.

Species-specific ways of detoxication and excretion of carcinogens--In a more general way, the chemical carcinogens can be considered cell toxins. There exist, of course, different ways of detoxification, particularly among the large number of species in the marine environment. Also the smaller phyla have been neglected here despite the fact that their use could reveal quite valuable information.

It is the main belief of the public that the cancer or the neoplasm in general is one type of disease, and the tumor itself is its focal point. In one respect this is true, but in another it is not, because the primary tumor is only one part of the process known as neoplastic disease.

MORPHOLOGICAL CHANGES AS A PARALLEL PROCESS OF CARCINOGENESIS IN DIFFERENT SPECIES USING SELECTED EXAMPLES

To complete our review, it is necessary to take a brief look at the morphological changes during carcinogenesis. We must keep in mind that biochemical changes and morphological changes are only two versions of the same process that run simultaneously but are observed with different methods. It was necessary to describe each separately.

The Change from Normal, Sometime Via Metaplastic, to Neoplastic Tissue in situ

The integument of many invertebrates is characterized by a simple columnar epithelium which is very often ciliated. The largest group of invertebrates, the insects, lack cilia. One of us (Kaiser) participated for many years in the investigation of the transformation of normal columnar ciliated epithelium of the human bronchus to its metaplastic stage to nonciliated squamous cell epithelium, and finally to the neoplasm *in situ*. As stated before, cilia are built similarly in nearly all organisms, plants and animals alike. We therefore selected the change in the columnar ciliated epithelium, which occurs during carcinogenesis.

The Development of the Neoplastic Tissue in situ to the Primary Neoplasm

The development of the neoplastic cells *in situ* is the first stage of the primary tumor and a crucial phase in the whole process of tumor development. It is the stage in which the barrier of basement membranes, in some cases, and the border to other tissues have to be broken. This means chemically a new adaptation. Simultaneously one of the main characteristics of the malignant neoplasms is established, that of infiltrative growth. It is also a turning point if compared with the characteristics of benign neoplasms, which lack the capability of infiltrative growth, and are generally characterized by a surrounding stroma capsule instead. On the other hand, it must be stated that the neoplastic cell *in situ* already has the cellular malignant characteristics.

Metastatic Developmenmt of Animal Neoplasms Only

For comparative purposes, we should take a short view of metastatic development and the most important group-specific differences. This is necessary to study and understand the various groups of marine organisms.

(1) All malignant plant tumors lack metastatic growth. This needs to be associated with two characteristic facts of plant cells and tissues:
    (a) The plant cell is characterized by a rigid cell wall.
    (b) No floating cells occur or are able to survive in the body fluid of vascular plants.

(2) Each metastatic growth in an organism is dependent on a circulatory system. Von Albertini (1974) distinguished between hematogenic and lymphogenic and three special types of metastasis in the human. In marine organisms, the hematogenic metastasis is the one of importance which, of course, will be modified by the type of circulatory in the different animal groups. Our knowledge of the manner in which tumors spread has increased (Kaiser, 1981, 1982). We also know more today about the multistep processes. Our expanded knowledge is also reflected in a better understanding of the sequence of the events in the metastatic process and its heterogeneity (Folkman, 1982; Fidler and Hart, 1982).

SUMMARY AND CONCLUSIONS

As we look backward and also forward, we see that neoplastic growth in organisms is a complex process which can be separated into several phases, seen from a morphological as well as a biochemical (metabolic) point of view. These different phases run over a species- and tumor-specific length of time. It is possible to distinguish the external and internal environments of living matter. The different portions of the external and internal environments interact with each other, as can be seen in certain characteristic chemical processes. Among the most important and best studied groups of chemical carcinogens are the polynuclear aromatic hydrocarbons. The pathways of their metabolism were studied mainly in terrestrial animals. The marine organisms invite additional study because the diversification of the specific structures and biochemistry could shed tremendous new insight. In addition, marine organisms are and can be excellent indicators of pollution in the largest environmental unit on earth, which is so important to the future of man and his environment. But to reach this goal, we must increase our knowledge of toxicants in the marine environment. The compounds of concern in this symposium are among the most significant and challenging groups. Of highest importance are the species-specific ways of transformation of these compounds in marine organisms as seen under comparative illumination. Participants in this symposium will inform us of the newest scientific research in the field, in the sense of Aristotle's kathekaston.

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NEGATIVE CHEMICAL IONIZATION MASS SPECTRA OF SOME POLYNUCLEAR AROMATIC HYDROCARBONS

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Abstract

Negative chemical ionization (NCI) mass spectra of 9 ortho-fused, 8 ortho and peri-fushed, and 13 methylated polynuclear aromatic hydrocarbons (PAHs) were obtained using isobutane as the reagent gas. The isobutane-mediated NCI (NCI-IB) spectra of all 30 PAHs were characterized by a small number of abundant, large-mass ions, suitable for qualitative or quantitative detection. In general, initial processes of resonance electron capture or anion attachment account for the principal ions formed by the unsubstituted PAHs, whereas dissociative capture resulting in loss of a hydrogen atom produces the most abundant ion in the NCI-IB mass spectrum of the alkylated PAHs. For most of the compounds studied, NCI-IB mass spectra produced in a hot (235°) ion source were better suited for high-sensitivity measurements than the corresponding spectra produced in a cooler (125°) source. NCI mass spectra do not provide information concurring isomeric structures, e.g., it would not be possible to distinguish benz(a)pyrene from benz(e)pyrene; however, the spectra reported indicate substantial potential for use of NCI mass spectrometry in screening environmental extracts for contamination with PAHs and their derivatives.

INTRODUCTION

Negative chemical ionization (NCI) mass spectra are obtained by operating a mass spectrometer with a high pressure (approximately one torr.) ion source in the negative ion mode. Spectra obtained under these conditions appear to be uniquely suited for screening extracts of environmental substrates for contamination with toxic substances. The reasons for this suitability stem from the fact that most man-made toxic substances are either oxidizing agents or alkylating agents, whereas most biomolecules are highly reduced and have large numbers of high energy electrons. Oxidizing or alkylating agents universally produce intense negative ion mass spectra, either through electron capture or through molecule-anion association. With the exception of free carboxcyclic acids and some of the prosphetic groups from the electron transport chain, biomolecules in contrast produce only very weak negative ion spectra. Thus it is possible to obtain molecularly specific information concerning the nature and the abundance of toxic substances in a matrix that contains a significant quantity of biomolecules in the extract (Dougherty and Piotrowska, 1967a, 1967b; Dougherty and Hett, 1978).

Negative chemical ionization mass spectra using methylene chloride as the reagent gas have been used to screen human urines and seminal fluid (Dougherty and Piotrowska, 1976a) and items from the food chain (Dougherty and Piotrowska, 1976b) for contamination with polychloroinated organics. NCI mass spectra can reliably detect polycyclic insecticides such as Mirex (Dougherty et al., 1976), industrial chemicals like polychlorobiphenyls (Dougherty et al., 1974), and polychlorodioxins (Hass et al., 1978) in extracts of environmental substrates.

Virtually all PAHs have positive electron affinities (Compton and Huebner, 1970) and should produce intense negative ion mass spectra. In order to increase the utility of NCI screening for toxic substances, we have obtained the NCI mass spectra of a series of polynuclear aromatic hydrocarbons using isobutane as the reagent gas. The use of hydrocarbon reagent gases is a simple extension of pressure enhanced negative ion mass spectra which was first applied to aromatic hydrocarbons (Hass et al., 1978).

The capture of thermal electrons occurs in low pressure mass spectrometers under conditions of electron impact ionization. This is generally a minor process at low source pressure because the reactions are sharply peaked at very low electron energies. Introduction of an inert gas or hydrocarbon gas to increase the pressure in the source can considerably enhance the population of low energy electrons and thus the formation of negative ions by resonance capture processes.

The capture of slow electrons is not only the process by which negative ions can be formed in a mass spectrometer. Electrons with energies between thermal energy and roughly ten electron volts are often captured by molecules with resulting fragmentation; the process is called "dissociative electron capture" (Reaction 2). The products of this reaction is an even-electron ion and a radical. Ionization of the reagent gas by resonance capture or disassociative capture may afford a population of anions within the source, and these anions may combine with the sample molecule to produce a negatively charged adduct (Reaction 3).

$$AB + e^{-}_{\text{slow}} \rightarrow AB^{\dot{-}*} \qquad (1)$$

$$AB + e^{-} \rightarrow A^{-} + B\cdot \qquad (2)$$

$$AB + C^{-} \rightarrow ABC^{-} \qquad (3)$$

Anions or molecule anions which contain considerable excess energy usually will be converted rapidly to neutrals by ejection of a electron. This means that the anions that one observes in NCI mass spectra must have low internal energies, which severely restricts the amount of fragmentation that can occur within the molecule. Thus negative ion mass spectra are essentially dominated by molecule anions, the products of disassociative capture, and anion-molecule reactions.

The ability of NCI mass spectra to specifically detect traces of halo-organics or PAHs in the presence of biomolecules is exemplified by the camparison of the isobutane-mediated NCI mass spectra of methylstearate and hexahelicene (Figure 1). Under our instrumental conditions, the isobutane mediated NCI mass spectrum of methylstearate contains no ions whatsoever at the highest sensitivity of the instrument. The NCI mass spectrum of helicene, on the other hand, contains essentially only one ion, the molecule anion, and the sensititivy of the technique is such that only one nanogram of hexahelicene is sufficient to produce a recognizable spectrum.

In this paper we report the hydrocarbon mediated NCI mass spectra for 30 different PAHs at two temperatures.

EXPERIMENTAL

NCI mass spectra were recorded using an AEI MS-902 double focussing mass spectrometer fitted with an SRIC chemical ionization source and an external-8 kV power supply. Research grade isobutane (Matheson) was introduced into the source through a needle valve at a rate adjusted to provide a pressure in the source of one torr (130 Pa); no effort was made to exclude adventitious oxygen. The source was maintained at 125 ± 5° for the first series of determinations and at 235 ± 5° for the second. PAHs were placed in freshly fired quartz cuvettes and introduced into the source on a direct insertion probe; heat was applied to those samples that did not vaporize spontaneously. Mass spectra were recorded using a light-beam oscillograph.

RESULTS AND DISCUSSION

Table 1 presents the principal ions in the isobutane-mediated negative chemical ionization mass spectra of the following polynuclear hydrocarbons at the source temperature: anthracene (1); phenanthrene (2); benzo(ghi)-fluoranthene (3); benz(a)anthracene (4); benzo(c)phenanthrene (5); chrysene (6); benzo(a)pyrene (7); benzo(e)pyrene (8); perylene (9); benzo(ghi)-perylene (10); benzo(b)triphenylene (11); dibenz(a,h)anthracene (12); coronene (13); dibenzo(def,p)chrysene (14); dibenzo(a,i)pyrene (15); hexahelicene (16); and dibenzo(g,p)chrysene (17).

TABLE 1. RELATIVE INTENSITIES OF PRINCIPAL IONS IN THE ISOBUTANE-MEDIATED NEGATIVE CHEMICAL IONIZATION (NCI-IB) MASS SPECTRA OF PAHs 1-17, DETERMINED AT SOURCE TEMPERATURES OF 125 ± 5° and 235 ± 5°

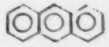
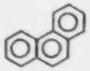
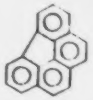
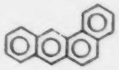
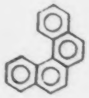
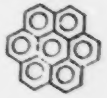
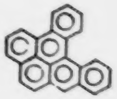
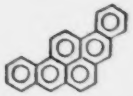
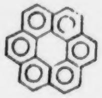
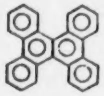
	PAH	Mol. Formula	$M^{\overline{\cdot}}$ (m/z; $\frac{I_{125°^a}}{I_{235°}}$)	$\overline{M-H}^-$ (m/z; $\frac{I_{125°^a}}{I_{235°}}$)	$\overline{MC-H}^-$ (m/z; $\frac{I_{125°^a}}{I_{235°}}$)	$MO_2^{\overline{\cdot}}$ (m/z; $\frac{I_{125°^a}}{I_{235°}}$)	$(M-H_2)^{\overline{\cdot}}$ (m/z; $\frac{I_{125°^a}}{I_{235°}}$)
(1)	 anthracene	$C_{14}H_{10}$	178; 100 100	177; 3 -	193; 19 19	210;10 -	
(2)	 penanthrene	$C_{14}H_{10}$	178; 55	177; 55	193; 100	210; -	
(3)	 benzo[ghi]fluoranthene	$C_{18}H_{10}$	226; 100 100	225; - -	241; 6 2	258; - -	
(4)	 benz[a]anthracene	$C_{18}H_{12}$	228; 100 100	227; - 4	243; 3 18	260; 42 2	
(5)	 benzo[c]phenanthrene	$C_{18}H_{12}$	228; 2 11	227; 10 34	243; 100 19	260: 55 3	226; 27 100

TABLE 1. (CONTINUED)

(13)	 coronene	$C_{24}H_{12}$	300; 100	299; 2	315; 21	332; -
(14)	 dibenzo[def,p]chrysene	$C_{24}H_{14}$	302; 100 100	301; - -	317; 3 2	334; - -
(15)	 dibenz[a,l]pyrene	$C_{24}H_{14}$	302; 100	301; -	317; 5	334; -
(16)	 hexahelicene	$C_{26}H_{16}$	328; 100 100	327; - 3	343; 10 12	360; 2 -
(17)	 dibenzo[g,p]chrysene	$C_{26}H_{16}$	328; 100 100	327; 2 12	343; 8 9	360; 2 1

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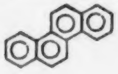
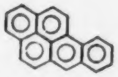
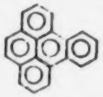
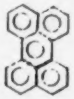
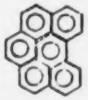
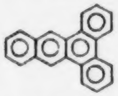
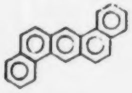
[a] Relative intensity at source temperature of 125 ± 5° and at 235 ± 5°, respectively; a dash indicates that the ion is not observed at significant intensity, and numbers represent percent of the intensity of the most-abundant ion (base peak).

TABLE 1. (CONTINUED)

(6)	 chrysene	$C_{18}H_{12}$	228; 14	227; 28	243; 100	260; -	226; 64
(7)	 benzo[a]pyrene	$C_{20}H_{12}$	252; 100 100	251; - -	267; - 3	284; - -	
(8)	 benzo[c]pyrene	$C_{20}H_{12}$	252; 100 30	251; - 28	267; 3 36	284; 92 4	250; - 100
(9)	 perylene	$C_{20}H_{12}$	252; 100	251; -	267; 7 -	284; -	
(10)	 benzo[g,h,i]perylene	$C_{22}H_{12}$	276; 100 100	275; - -	291; 5 3	308; - -	
(11)	 benzo[b]triphenylene	$C_{22}H_{14}$	278; 100 100	277; - 34	293; 2 72	310; 54 -	
(12)	 dibenz[a,h]anthracene	$C_{22}H_{14}$	278; 100 100	277; 13 4	293; 19 25	310; 45 3	

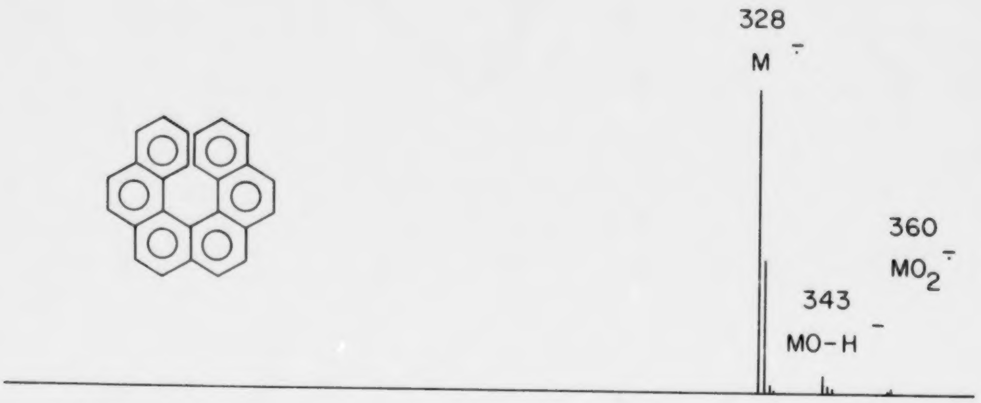


Figure 1. Isobutane mediated NCI mass spectrum of hexahelicene (16), measured at a source temperature of 120°.

The spectrum of hexahelicene (16, Figure 1) is typical of the members of this class, resonance captre (Reaction 1) produced the most abundant ion ($M^{\cdot-}$) at m/z 328; less-abundant ions form by capture of an electron and an oxygen molecule (Reactions 1 and 3) to give $MO_2^{\cdot-}$, m/z 360, which subsequently loses the elements of an hydroxyl radical to form a fragment ion that may be represented as an oxy analogue of the parent PAH, $(M-H+O)^-$, m/z 343. Dissociative capture (Reaction 2) contributes negligibly to the net ionization, even at the higher source temperature. Compounds 4,5,8,11, and 12 exhibit a much stronger tendency to form $MO_2^{\cdot-}$ in the cool source, thereby decreasing the amount of $M^{\cdot-}$ in the spectrum. Compounds 2,11, and 13 appear to be relatively inefficient at capturing electrons and gave relatively weak responses. Compounds 2,5, and 6 did not produce major molecule anions, although each did form one principal ion which was characteristic of the particular PAH and suitable for quantitative measurement. The mass spectrum of 12 also contained a metastable ion at m/z 249.5, suggesting that at least a fraction of the M^- ion (m/z 278) formed by loss of an oxygen molecule from $MO_2^{\cdot-}$ (m/z 310), which presumably formed by capture of $O_2^{\cdot-}$ (Reaction 3).

Table 2 presents the masses and relative intensity data for the principal ions in the isobutane NCI mass spectra of the following methylated PAHs: 4,5-dimethylphananthrene (18); 4-methylpryene (19), 3,4,5,6-tetramethylphenanthrene (20); 1-methylbenzo(c)phenanthrene (21); 2-methylbenzo(c)phenanthrene (22); 3-methylbenzo(c)phenanthrene (23); 4-methylbenzo(c)phenanthrene (24); 5-methylbenzo(c)phenanthrene (25); 6-methylbenzo(c)phenanthrene (26); 7,12-dimethylbenz(a)anthracene (27);

TABLE 2. RELATIVE INTENSITIES OF PRINCIPAL IONS IN THE ISOBUTANE-MEDIATED NEGATIVE CHEMICAL IONIZATION (NCI-IB) MASS SPECTRA OF PAHs 18-30, DETERMINED AT SOURCE TEMPERATURES OF 125 ± 5° amd 235 ± 5°

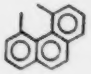
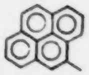
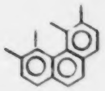
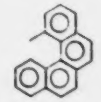
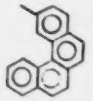
	PAH	Mol. Formula	$M^{\overline{\cdot}}$ (m/z; $\frac{I_{125°^a}}{I_{235}}$)	$M\text{-}H^-$ (m/z; $\frac{I_{125°^a}}{I_{235}}$)	$MO\text{-}H^-$ (m/z; $\frac{I_{125°^a}}{I_{235}}$)	$MO_2^{\overline{\cdot}}$ (m/z; $\frac{I_{125°^a}}{I_{235}}$)	Other Ions
(18)	 4,5-dimethylphenanthrene	$C_{16}H_{14}$	206; 13	205; 49	221; 100	238; 21	
(19)	 4-methylpyrene	$C_{17}H_{12}$	216; 46 19	215; 100 100	231; 40 10	248; 76 4	
(20)	 3,4,5,6-tetramethylphenanthrene	$C_{18}H_{18}$	234; 39 22	233; 100 100	249; 50 10	266; 37 -	
(21)	 1-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 23 27	241; 100 100	257; 47 32	274; 34 9	297; - 53
(22)	 2-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 12 40	241; 57 100	257; 26 29	274; 100 -	297; - 56 272; - 231

TABLE 2. (CONTINUED)

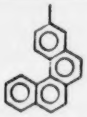
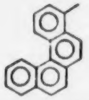
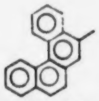
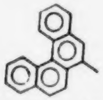
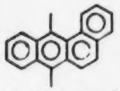
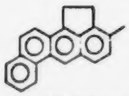
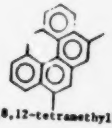
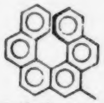
(23)	 3-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 62 69	241; 74 100	257; 25 53	274; 100 -	297; 20 5	272; 1 163
(24)	 4-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 25 19	241; 100 100	257; 82 25	274; 97 7	297; 31 -	272; - 20 256; 26 23
(25)	 5-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 25 21	241; 100 100	257; 42 12	274; 48 11	290; 18 -	256; 10 14
(26)	 6-methylbenzo[c]phenanthrene	$C_{19}H_{14}$	242; 21 24	241; 100 100	257; 90 19	274; 63 13	297; 40 -	256; 19 22
(27)	 7,12-dimethylbenz[a]anthracene	$C_{20}H_{16}$	256; 100 100	255; - 71	271; 1 13	288; - 2		
(28)	 3-methylcholanthrene	$C_{21}H_{16}$	268; 14 4	267; 100 27	283; - 2	300; - -	266; 92 100	

TABLE 2. (CONTINUED)

(29)	 1,5,8,12-tetramethylbenzo[c]phenanthrene	$C_{22}H_{20}$	284; 28 31	283; 100 100	299; 27 23	316; 39 20	
(30)	 7-methylhexahelicene	$C_{27}H_{18}$	342; 32 100	341; 100 20	357; 11 14	374; 2 3	340; 21 20

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[a] See footnote a, Table 1.

[b] Line(s) projecting from the aromatic nucleus indicate the location of the methyl substituent(s).

3-methlcholanthrene (28); 1,5,8,12-tetramethylbenzo(c)phenanthrene (29); and 7-methylhexahelicene (30), at source temperatures of 125 ± 5° and 235 ± 5°. The spectrum of 7-methylhexahelicene (30) is typical of that of most members of this group, having as the base peak the product of dissociative capture (Reaction 2) with loss of hydrogen atom to give the ion represented as $[M-H]^-$, at m/z 341. Most of the signal observed at m/z 342 derives from $[M-H]^-$ ions containing a ^{13}C aton and only a small amount of the molecular anion ($M^{\cdot-}$) is formed. $MO_2^{\cdot-}$ and $(M-H+O)^-$ are also minor ions in the spectrum. The mass spectrum of 30 was relatively insensitive to the temperature of the ion source.

Conversely, the spectra of the isomeric methylbenzo(c)phenanthrenes (21-26) illustrate a striking dependence on source temperature. At 235°, the spectra of all six isomers are substantially identical, whereas the corresponding spectra measured at 125° exhibit gross variations in relative intensitites for different isomers. The latter observation is of potential interest for structural studies, but for analytical purposes, a spectrum in which a single, characteristic ion preponderates offers greater sensitivity.

The $(M-H)^-$ ion was comparatively minor in the NCI spectra of 18, 27, and 28, although each gives one major ion. Loss of H_2 from M^- occured extensively in 28, the $(M-2H)^-$ ion being the base peak in the hot source. Dehydrogenation of 28 would yield a PAH containing a cyclopentadieneide ring which should be a very stable anion. The higher temperature spectrum of 27 exhibited a large increase in the intensity of $(M-H)^-$, which would be expected if the disassociative capture reaction were thermally activated.

The relative amount of $MO_2^{\cdot-}$ was observed to decrease as the sample remained in the ion source. The use of a reagent gas mixture containing a fixed proportion of oxygen stabilized the relative proportions of products, e.g. $MO_2^{\cdot-}$, formed subsequent to ionization.

Two other molecules examined in this study of these picene failed to produce ions presumably because of their involatility. At either source temperature, the NCI spectrum of triphenylene (M.W. 228) produced only m/z 252, which cannot be accounted for as simply as the ions formed from 1-30, but is nonetheless quite suitable for quantitative detection of trace amounts of triphenylene.

CONCLUSIONS

The isobutane NCI mass spectra of 30 PAHs show real promise for the development of screening methods of these compounds in environmental substrates. The spectra were uniformly intense-roughly nonogram sample requirements and molecularly specific. The spectra of alkylated PAHs were generally dominated by $(M-H)^-$ ions while those of the parent hydrocarbons were dominated by molecule anions.

ACKNOWLEDGEMENTS

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A CHARACTERIZATION OF THE POLYCYCLIC AROMATIC HYDROCARBON CONTENT OF TARS, TARBALLS, AND SEDIMENTS FROM THE MARINE ENVIRONMENT

by

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ABSTRACT

Glass capillary column gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were used to characterize the hydrocarbons present in samples of tars, tarballs, and sediments from the marine environment. Emphasis was directed toward the analysis of polycyclic aromatic hydrocarbons (PAHs) because of their known toxicity. The relative abundances of alkylated vs non-alkylated PAHs and GC patterns obtained from the tarballs indicated that tarball samples collected on the Brittany Coast of France were petroleum-derived, whereas those obtained on a Rhode Island beach were formed from coal tar.

Analyses of PAHs in sediments and in coal tar from docks included determinations of (1) the relative content of non-alkylated PAH parent molecules, i.e. parent compound distributions (PCDs); (2) alkylation patterns of these PAH molecules, i.e., alkyl homolog distributions (AHDs); and (3) phenanthrene/anthracene (P/A) ratios. Comparisons of these measurements demonstrated that the sediments surrounding tarred piers were contaminated by coal tar used to coat the pilings; however, the PAH assemblages in samples of sediment from the middle of Narragansett Bay reflected a different source.

INTRODUCTION

Concern about the toxicity of polycyclic aromatic hydrocarbons (PAHs) has resulted in research to determine the sources and fates of these compounds in the environment. Researchers have characterized PAH assemblages and investigated the spatial distribution of PAH compounds in samples of sediments from the marine environment (Youngblood and Blumer, 1975; Hites and Biemann, 1975; Hase and Hites, 1976; Hites, 1976; Hites et al., 1977;

Farrington et al., 1977; LaFlamme and Hites, 1978; Windsor and Hites, 1979). These studies found that the PAH assemblages in marine sediments contained numerous non-alkylated (parent) PAH compounds and the alkyl homologs of these parent compounds. The PAH alkyl homolog distributions found in marine sediments have been compared with those in airborne particulate material (Hase and Hites, 1976), combustion products of fossils fuels (Lee et al., 1977; Hase and Hites, 1976), petroleum (Youngblood and Blumer, 1975), and water (Hase and Hites, 1976). Studies have investigated the probable origins of PAHs found in marine sediments in New England (Windsor and Hites, 1978; Lake et al., 1979) and in other areas (LaFlamme and Hites, 1978). The determination that the PAH content of marine sediments rapidly decreased with increased distance from population centers (Windsor and Hites, 1979; Lake et al., 1979) indicated that most of the PAHs in these sediments came from anthropogenic inputs.

While the majority of PAHs present in marine sediments were believed to result form the deposition of aeolian transported fossil fuel combustion products (LeFlamme and Hites, 1978), or in some instances, from a combination of inputs from combustion and petroleum (Lake et al., 1979), smaller localized inputs of PAH may have environmental significance. To further characterize PAH inputs to the marine environment, we examined the hydrocarbon contents of tarballs, coal tar used in marine construction, and marine sediments.

METHODS

Samples of sediments taken near piers in the Narragansett Bay and in New Harbor on Block Island, RI, were collected with a Ponar grab sampler. The top 10 cm of an "undisturbed" portion of the sediment were retained for analysis. A sediment sample was obtained near the north end of Jamestown Island in mid-Narragansett Bay by a diver using a plexiglas core tube.

All sediment samples were returned to the laboratory as quickly as possible and analyzed immediately or frozen at -20° C until analysis. Prior to analysis of the top 10 cm section of the core, the sediment contacting the core liner was scraped off.

The extraction and clean-up procedures used on sediment samples have been described (Lake et al., 1980). Briefly, this method consists of a methylene chloride reflux extraction followed by separation of the extract into F-1 (aliphatic hydrocarbons, including some olefins) and F-2 (aromatic hydrocarbons, including some olefins and PAH compounds) fractions on a silica gel column.

Samples of tarballs were collected on the Narragansett Town Beach during May of 1978 and on the Brittany Coast of France* in March 1978. Samples of coal tar used to coat docks were scraped from piers.

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*The tarballs from the Brittany Coast were well-aged and did not originate (or were not contaminated) during the AMOCO CADIZ oil spill.

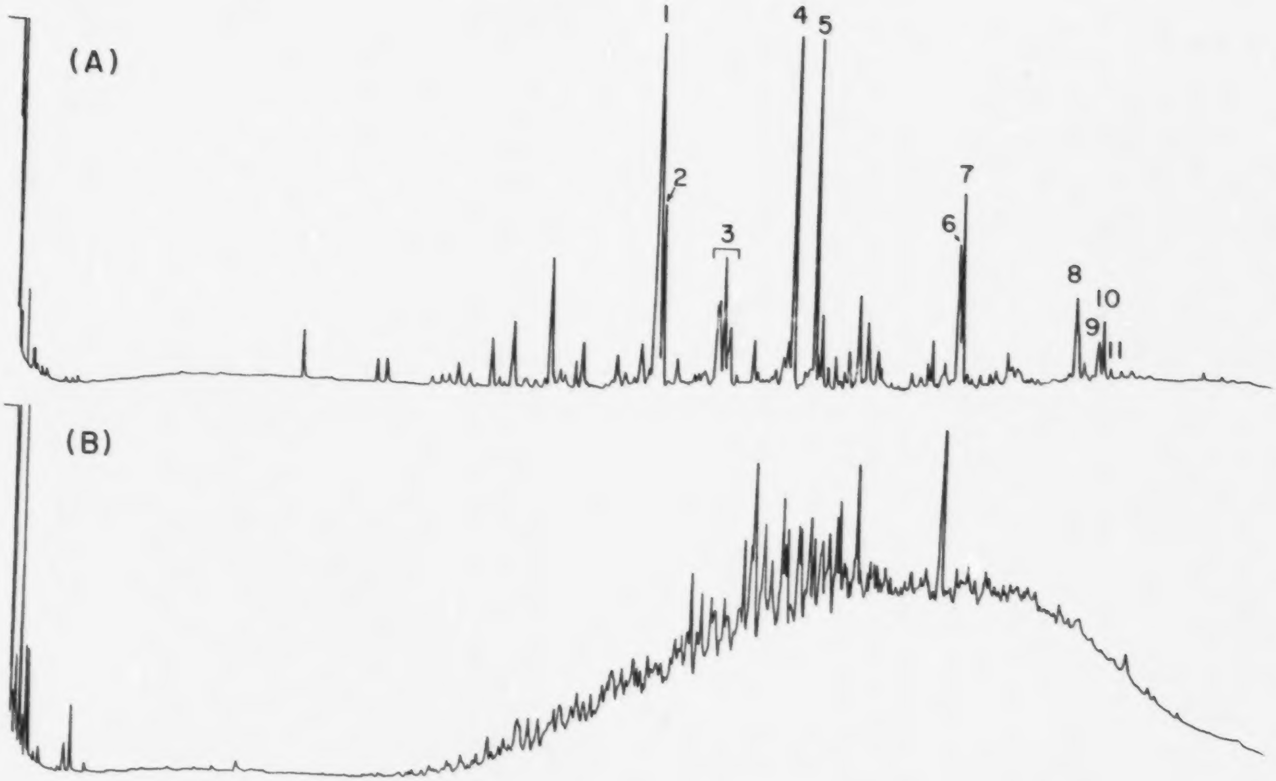

Figure 1. Gas chromatograms of F-2 fraction of: (A) tarball sample from Narragansett Beach, (B) tarball sample from Brittany Coast of France.

TABLE 1. PAH COMPOUNDS IN FIGURE 1

Compound Number	Molecular Weight	Z Number	Tentative Compound Identification
1	178	-18	Phenanthrene
2	178	-18	Anthracene
3*	192	-18	C_1-Phenanthrenes + C_1-Anthracenes
4	202	-22	Fluoranthene
5	202	-22	Pyrene
6	228	-24	Benzanthracene
7	228	-24	Chrysene
8	252	-28	Benzofluoranthene
9	252	-28	Benzo(e)pyrene
10	252	-28	Benzo(a)pyrene
11	252	-28	Perylene

* Other compounds may also be present under this bracket.

Tars and tarballs were dissolved in $CH_2\ Cl_2$, solvent exchanged to hexane and separated into F-1 and F-2 fractions on silica gel columns.

The column chromatographic fractions were analyzed on glass capillary columns by both gas chromatography and gas chromatography-mass spectrometry (GC-MS). The method of analysis has been described in detail (Lake et al., 1980). This analysis determined the concentrations of selected PAH compounds by integrating the GC-MS extracted ion current profiles (EICPs) corresponding to the molecular ions of the compounds of interest. Specific peaks in the EICPs were not integrated if their spectra did not correspond to those of the compounds of interest or if they did not have characteristic retention times. The integrated values were normalized and displayed in a semi-logarithmic format as parent compound distributions (PCDs) and alkyl homolog distributions (AHDs). PCDs represent the relative concentrations of the parent compounds of interest; i.e., PAHs with molecular weights of 178, 202, 228, 252, 276, and 278. These distributions were obtained by correcting the raw data for instrument response with the aid of response factors calculated from known PAH standards. In the few instances where a standard was not available, raw data were corrected by the use of another PAH standard with the same molecular weight. AHDs show the concentration of the parent compound in relation to its C_1 through C_3 alkyl homologs. The AHDs were not corrected for instrument response because of a lack of necessary alkylated standards. Z numbers were calculated from C_nH_{2n+Z}. Phenanthrene/anthracene (P/A) ratios were calculated by integrating separately the areas under the two peaks in the EICP for m/e of 178.

The GC analyses were performed on a 20 m glass capillary column coated with SE-54 in a Hewlett-Packard 5840A GC with splitless injection. The GC-MS analyses were performed on a similar column in a Shimazdu Model GC-4CM GC connected to a Finnegan 1015 mass spectrometer equipped with a Systems Industries data system with Riber D-8 software. The mass spectrometer was operated in the EI (electron impact) mode at 70 eV. Reagent blanks and standard samples were processed with samples to ensure continued satisfactory performance of the methods.

RESULTS

The gas chromatograms (GCs) of fraction 1 (F-1) of the tarballs collected from Narragansett Beach showed only small amounts of hydrocarbon material whereas, GCs from the F-1 fraction of the French tarball showed a much higher proportion of normal and branched alkanes in addition to a large area under the resolved peaks called the "unresolved complex mixture." GCs from the F-2 of the Narragansett tarballs were well-resolved. The major peaks in these fractions were non-alkylated PAH molecules (Figure 1(A); Table I). In contrast, GCs of F-2 fractions from the French tarballs were not well resolved (Figure 1(B)). GC-MS analyses of the F-2 fractions from the French tarballs revealed that many of the compounds were alkylated compounds of the Z=-12 (naphthalene) and the Z=-18 (anthracene/phenanthrene) homolog series.

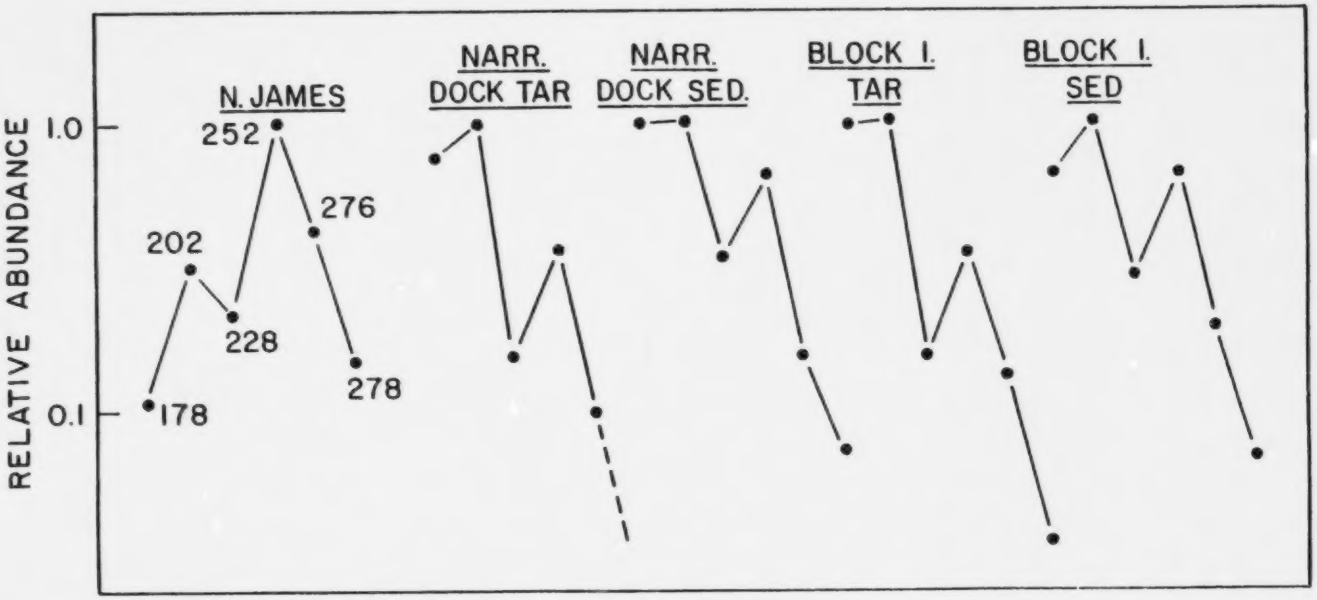

Figure 2. Parent Compound Distributions (PCDs) from samples of tars and sediments.

The gas chromatograms of extracts from sediments surrounding docks from Narragansett Bay and Block Island, Rhode Island, showed relatively small amounts of material in the F-1 fractions, but relatively large amounts of well resolved compounds in the F-2 fractions. Similar GCs were obtained from extracts of coal tar collected from docks. GC-MS analyses of F-2 fractions from these samples showed that most of the largest resolved peaks in these chromatograms were non-alkylated PAHs. The PCDs and Z=-22 AHDs of representative samples are shown (Figures 2 and 3). The phenanthrene/anthracene (P/A) ratios of these samples were relatively high (Table 2).

The GCs from the sediment sample collected near Jamestown Island showed a large unresolved complex mixture in the F-1 fraction, but larger relative amounts of resolved components in the F-2 fraction. GC-MS analysis of the F-2 fraction showed some of the resolved compounds were PAHs. The PCDs and Z=-22 AHDs calculated from this sample are shown (Figures 2 and 3). The P/A ratio of this sample was relatively low (Table 2).

DISCUSSION

Analyses of tarballs from Rhode Island and from the Brittany Coast of France revealed major differences in chemical composition. Gas chromatograms (GCs) of the F-1 fractions showed that the tarballs from Narragansett Beach contained relatively small amounts of both resolved normal alkanes and unresolved complex mixture. In contrast, French tarballs contained relatively large amounts of normal alkanes, the isoprenoids pristane and phytane, and a large amount of unresolved complex material.

GCs from the F-2 fraction from the Narragansett tarball showed well resolved non-alkylated PAH compounds (Figure 1(A), Table I), and were very similar to GCs obtained from coal tar collected from dock coatings. The GCs from the F-2 fractions of French tarballs were not well resolved (Figure 1(B)). These fractions contained many alkylated compounds in the Z=-12 (naphthalene) and Z=-18 (anthracene/phenanthrene) homolog series.

The relatively low amount of material in F-1 fractions, the abundance of well-resolved, non-alkylated PAH compounds in F-2 fractions, and the similarities of GCs from Narragansett tarballs and those from coal tar used to coat pilings indicates that the tarball samples from Narragansett Beach were probably coal tar. Comparisons of the GCs of F-1 fractions from the French tarball samples with GCs from some petroleum derived tarballs (Morris and Butler, 1973) showed many similarities in the patterns of alkane peaks and in the relative amount of unresolved complex mixture. These similarities and the French tarballs' high content of alkylated PAH compounds in the Z=-12 and Z=-18 homolog series, which are the most abundant PAHs found in petroleum (Pancirov and Brown, 1975; Youngblood and Blumer, 1975), combined to indicate that the tarball samples from the Brittany Coast probably originated from petroleum.

An examination of the PAH assemblages in extracts of sediments obtained near piers in the Narragansett Bay was undertaken to determine if the coal tar used to coat these piers was a significant source of PAHs to Narragansett Bay sediments. Comparisons of PCDs, AHDs, and P/A ratios from the sediments near the docks with those obtained from the coal tar used to coat the docks showed a definite correspondence between the PAHs in the dock tar and in the sediments. The PAH assemblages in the dock tar and in these contaminated sediments showed characteristic PCDs (Figure 2) steeply sloping Z=-22 AHDs (Figure 3), and relatively high (>20) P/A ratios (Table 2). To ensure that other inputs of PAH material were not influencing the observed measurements, samples of dock tar and sediments were obtained from a relatively pristine location-Block Island, Rhode Island (14 miles off the coast of Rhode Island). The PAH measurements from these samples matched each other and corresponding tar and sediment samples from Narragansett Bay. These data indicate that the use of coal tar in marine construction resulted in the input of PAH compounds to sediments adjacent to docks.

The PCD, AHD, and P/A ratio of the North Jamestown sediment sample are shown in Figures 2 and 3 and in Table 2. The PAH assemblage of this sample has been shown to be quite similar to those in other Narragansett Bay sediments which were not collected near docks (Lake et al., 1979). Comparisons of the above measurements with those from coal tar used to coat the docks showed many differences and thereby indicated that dock tar was probably not the major PAH contaminant of bay sediments. Rather, as described in detail elsewhere (Lake et al., 1979), the PAH assemblages in these sediment samples appeared to result from a combination of inputs.

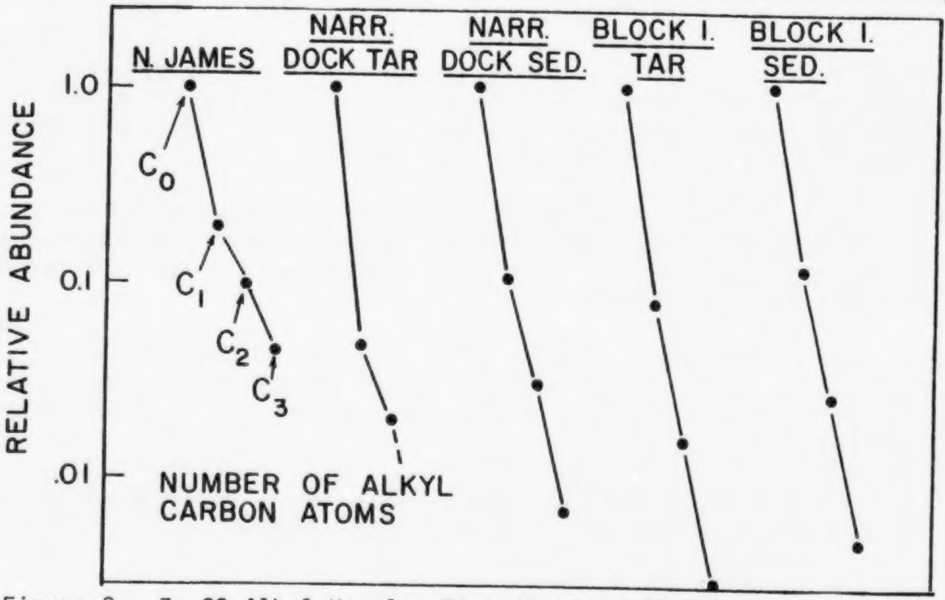

Figure 3. Z=-22 Alkyl Homolog Distributions (AHDs) from samples of tars and sediments.

TABLE 2. PHENANTHRENE/ANTHRACENE RATIOS OF DOCK TARS AND SEDIMENTS

Description of Sample	P/A Ratio
Narragansett Dock Tar	38
Block Island Dock Tar	39
Narragansett Dock Sediment	26
Block Island Dock Sediment	23
North Jamestown Sediment	4.3

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TOXIC PHOTOOXYGENATED PRODUCTS GENERATED UNDER ENVIRONMENTAL CONDITIONS FROM PHENANTHRENE

by

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ABSTRACT

The photooxidation of phenanthrene under simulated environmental conditions to 9,10-epoxy-9,10-dihydrophenanthrene, among other oxygenated products, serves as a possible model for the conversion of polycyclic aromatic hydrocarbons (PAHs) to potentially mutagenic and/or carcinogenic products in the environment. The reaction was carried out in a hexane-aqueous phase illuminated by a lamp whose output is similar to that of sunlight. These toxic photoproducts were isolated and identified by glass capillary gas chromatography-mass spectrometry, through comparison of GC retention times and mass spectral fragmentation patterns with data observed for authentic samples obtained independently through synthesis or commercial sources. Some of these products were found to be soluble in water which suggests the possibility of the intrusion of oxygenated PAHs into natural waters. These results are discussed in terms of the environmental oxidative processes of various mutagenic and carcinogenic PAHs.

INTRODUCTION

A recent study of 22 PAHs reveals that the PAHs themselves are not mutagenic towards *Salmonella typhimurium*, but their irradiated reaction mixtures are found to be mutagenic (Gibson *et al*., 1978). In general, PAHs that are mutagenic or carcinogenic require metabolic activation before they can induce their effect (Asby and Styles, 1978). Although the mutagenicity of some PAHs appears to be involved by both photooxidation by air and oxidation by rat liver S9 microsomes, they may operate by different or identical oxidative mechanisms to generate oxygenated products (Gibson *et al*., 1978). The biotransformation of PAHs to mutagens or carcinogens needs further study as well as chemical identification of active metabolites. The oxygenated products of various PAHs such as epoxides, phenols, diols, and epoxydiols present a threat to human health as evidenced in several

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hundred toxicological reports on their carcinogenicity and mutagenicity (DePierre and Ernster, 1978; Yang et al., 1978).

Among the widely claimed environmental sources containing PAHs are urban air (Bartle et al., 1977; Bjorseth, 1977; Giger and Schaffner, 1978; Smillie et al., 1978), tobacco smoke (Bartle et al., 1977), coal tar (Lijinsky et al., 1963), coal (Schabron et al., 1977; Woo et al., 1978), soot of electrolysis furnaces (Tausch and Stehlik, 1977), marijuana smoke (Bartle et al., 1977), mineral oils (Lijinsky et al., 1963), petroleum (Lijinsky et al., 1963), sediments (Giger and Schaffner, 1978; Jungclaus et al. 1978), river particulates (Giger and Schaffner, 1978), sludge (Grimmer et al., 1978), tissues of marine organisms (Pancirov and Brown, 1977), industrial waste waters (Jungclaus et al., 1978), street dust (Giger and Schaffner, 1978, and even drinking waters (Basu and Saxena, 1978). Recently, the ocean has been contaminated by oil spills which deposit tons of petroleum hydrocarbons (Boylan and Tripp, 1971). It has been noted, however, that photooxidative processes presumably occurring at the surface of the oil films can lead to other highly toxic and water soluble materials during such weathering processes (Scheier and Gominger, 1976). The leached water soluble oxygenated products can be toxic to algae, fish, and other marine organisms. Recent evidence (Lacaze and de Naide, 1976; Payne et al., 1978; Scheier and Gominger, 1976) indicates increased toxicity of the water soluble fraction after irradiation of crude oils. Thus photooxidation of petroleum hydrocarbons in the environment increases the toxicity of petroleum and may present a threat to human health and have an effect on marine organisms. The natural seepage of oil and PAHs onto the continental shelf has been estimated to release 0.2×10^6 to 6×10^6 metric tons of PAHs per year into the sea (Wilson et al., 1974). The finding that the ratio of aromatic hydrocarbons to cycloalkanes decreases in non-volatile dispersed oil on the sea surface suggests that aromatic hydrocarbons are destroyed by sunlight. Polycyclic aromatic hydrocarbons (1 to 3.5% of crude oils) may be oxidized in nature by the molecular oxygen in air and sunlight or by singlet oxygen present in natural waters (Zepp et al., 1977), or produced by molecular oxygen and naturally occurring hydrocarbons which act as photosensitizers (Patel et al., 1978c). Phenanthrene and several other aromatic and heteroaromatic hydrocarbons commonly found in petroleum have been observed to be decomposed by irradiation (Nagata and Kondo, 1977).

The ultimate mutagenic or carcinogenic activity of PAHs is coupled through the metabolic activation to primary and secondary metabolites. Dihydroepoxides of PAHs have been the subject of extensive studies of metabolic carcinogens, and their interaction with DNA has also been investigated (Sims and Grover, 1974). Attention has been focused on 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene and its role as the ultimate carcinogen of benzo(a)pyrene (Yang et al., 1978). However, there is widespread evidence that other polycyclic aromatic hydrocarbon epoxides behave similarly (Sims and Grover, 1974). It is of interest to know that photo- oxidation is the most significant mechanism by which the carcinogenic benzo(a)pyrene degrades in aqueous systems in the presence of oxygen (Suess, 1977). This strongly suggests a need to investigate the effects of photooxidation on other polycyclic aromatic hydrocarbons commonly found in crude oils. The formation of proximate carcinogens in the environment through photooxidation or biological pathways is an important though disturbing possibility which merits further study.

We have carried out the photooxidation of such PAHs under simulated environmental conditions and have isolated some of the major photoxygenated products which are known to be toxic, carcinogenic, and mutagenic. This report presents the isolation and the characterization of such products from phenanthrene, a model PAH contaminant. Moreover, phenanthrene is a relatively simple PAH containing both a "K-region" and a "bay-region"; molecular features of certain PAHs have been the object of considerable investigation with respect to the carcinogenicity of those compounds.

METHODS AND MATERIALS

(A) Photooxidation and Separation of Products

A two-phase system composed of solutions of phenanthrene in hexane on synthetic seawater (pH 8.2) was irradiated at the surface as previously described (Dowty *et al.*, 1974) under conditions simulating those found in the environment at air-sea interfaces (sea-water droplets formed by wind and waves at the sea surface [Bezdek, and Carlucci, 1974]). A visible source (Sylvania EHC 500 watt tungsten lamp shielded by a Uranium glass filter) whose spectral output approximates that of sunlight was employed*. The reactions were conducted in the presence of oxygen and perylene, a naturally occurring PAH (Lijinsky *et al.*, 1963; Schabron *et al.*, 1977; Giger and Scaffner, 1978; Smille *et al.*, 1978; Woo *et al.*, 1978), which acts as a singlet oxygen sensitizer.

The reaction mixtures obtained upon photooxidation were separated into two fractions (hexane and aqueous) (Figure 1). The hexane fraction was dried over anhydrous sodium sulfate and concentrated under vacuum on a rotary evaporator, while the aqueous fraction was extracted with dichloromethane. The remaining aqueous layer was made acidic by the addition of 1 M hydrochloric acid (pH 2) and then extracted with dichloromethane. The dichloromethane extract containing the acidic components (mainly phenols and carboxylic acids) was methylated with methyl iodide and sodium hydroxide in dimethyl sulphoxide (CH_3SOCH_3) according to the method described by Gillis (1968). The photooxidation of 9,10-epoxy-9,10-dihydrophenanthrene and other oxygenated products were conducted under conditions similar to those mentioned for phenanthrene. The obvious control experiments conducted in the dark, in the absence of sensitizer and without oxygen, were also carried out under otherwise

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*The absorbance spectra of uranium glass probe measured on a Model Cary 17 spectrophotometer cuts out at 339.5 nm, i.e., ultraviolet radiation was blocked out.

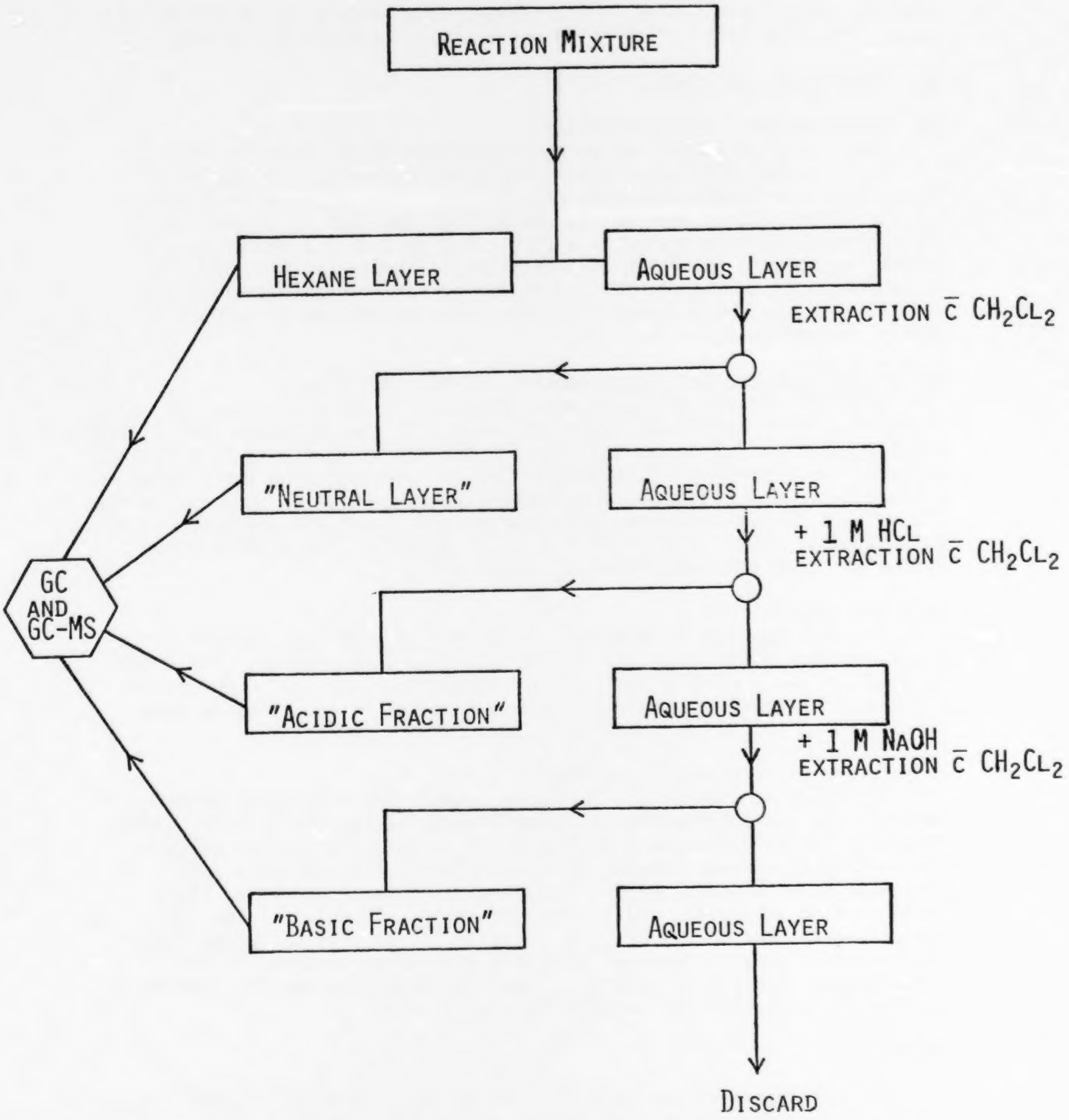
REACTION MIXTURE
HEXANE LAYER
AQUEOUS LAYER
EXTRACTION $\bar{c}$ CH_2Cl_2
"NEUTRAL LAYER"
AQUEOUS LAYER
+ 1 M HCL
EXTRACTION $\bar{c}$ CH_2Cl_2
GC AND GC-MS
"ACIDIC FRACTION"
AQUEOUS LAYER
+ 1 M NAOH
EXTRACTION $\bar{c}$ CH_2Cl_2
"BASIC FRACTION"
AQUEOUS LAYER
DISCARD

Figure 1. Experimental diagram of the photooxygenated reaction mixture into compound-class fractions.

identical conditions. Finally the hexane, dichloromethane, and methylated acidic fractions were concentrated and analyzed by GC-MS techniques.

(B) Analytical Procedures

Gas Chromatographic (GC) Analysis--

Products were identified by the comparison of GC retention times. All analyses used a Hewlett-Packard 5711A gas chromatograph coupled to a Hewlett-Packard 3354A Laboratory Data System. The injection port and detector temperatures were maintained at 250° and 300° C, respectively. The compounds were separated in a 28 m x 0.3 mm (i.d.) glass capillary column coated with SE-52 (in dichloromethane) (Lawler *et al*., 1977). Helium was used as the carrier gas at a flow rate of approximately 1.9 mℓ/min. The air and hydrogen flow rates were maintained at 240 and 30 mℓ/min, respectively.

Gas Chromatographic-Mass Spectrometric (GC-Ms) Analysis--

All products and reactants were subjected to analysis using the same chromatrographic conditions described above with the exception that a glass capillary column (13 m) was utilized and the carrier gas flow rate was adjusted correspondingly (3 mℓ/min). The gas chromatograph was coupled to a Hewlett-Packard 5980A mass spectrometer operated with an ionizing voltage (70 eV) and 160 μamp current. The source temperature and the pressure were maintained at 150° C and 2×10^{-5} Torr. A Hewlett-Packard 5933A Data System was interfaced with the mass spectrometer.

Mass Spectral Analysis--

The mass spectral fragmentation patterns of authentic reference standards were obtained by the direct insertion probe method. The probe temperature was varied between ambient temperature and 150° C during the course of the analyses, while the source temperature and pressure were maintained at 155° C and 6.4×10^{-8} Torr.

Preparation of Reference Standards--

Authentic samples of the following products were prepared by methods described in the literature: 2'-formylbiphenyl-2-carboxylic acid (Bailey, 1956), 2,2'-diformylbiphenyl (Bailey and Erickson, 1961), 9,10-epoxy-9,10-dihydrophenanthrene (Newman and Blum, 1964; 2,3:4,5-dibenzoxepin (Brightwell and Griffin, 1973), 9-phenanthrol (Newman and Blum, 1964), diphenic acid anhydride, and 3,4-benzocoumarin. (Samples of the latter two products were provided by Dr. G.W. Griffin.) The physical properties of the synthetic samples compared favorably with those reported in the lieterature. Additional reference standards were obtained from commercial sources (Aldrich Chemical Company, Milwaukee, WI).

RESULTS

The air-sea interface model created by oxygen bubbled through a two-phase system (composed of a phenanthrene solution in hexane on synthetic seawater) was found to be appropriate for the photooxidation of PAHs under simulated environmental conditions. The direct exposure of sunlight and atmospheric oxygen in the marine environment is most likely to occur at the air-sea interface where tiny water droplets are formed by the action of wind and waves (Bezdek and Carlucci, 1971). The UV-visible spectra measured for the visible source in our study (Sylvania EHC 500 watt tungsten lamp covered by a Uranium glass filter) approximates that of sunlight.

Among the various photosensitizers evaluated, the naturally occurring hydrocarbon, perylene (Lijinsky et al., 1963; Schabron et al., 1977; Giger and Schaffner, 1978; Smillie et al., 1978; Woo et al., 1978), was found to be an effective photosensitizer for the generation of the different classes of oxygenated products from phenanthrene (Patel et al., 1978c). The results of these reactions, i.e., the product ratios, were compared with those observed in the photooxidation of phenanthrene conducted with known photosensitizers such as methylene blue and rose bengal. The results were identical, which indicates that a naturally occurring organic compound can lead to the sequences of events involving singlet oxygen, resulting in the photodecomposition of aromatic hydrocarbons.

The disappearance of phenanthrene was monitored by analyzing aliquots collected at intermittent time intervals (0 to 22 hr) as the reaction progressed (Figure 2). During the course of the experiment, 32% of the phenanthrene was consumed. The halflife of the phenanthrene under these conditions was determined to be approximately 80 hr.

To improve GC resolution and facilitate identification of the major products obtained upon photooxidation of phenanthrene, the acidic products (mainly carboxylic acids and phenols) were effectively separated initially by liquid-liquid extraction from the aqueous phase. The acidic components were subsequently methylated utilizing methyl iodide prior to analysis (Gillis, 1968)(Figure 3D). Peaks are identified in Table 1, and the structures of corresponding compounds are shown in Figure 4.

The structures of the individual components were established by comparing GC retention times and mass spectral fragmentation patterns with the corresponding data obtained with authentic samples. The precise mass spectral fragmentation patterns of the primary and secondary photoproducts of phenanthrene were determined (Patel et al., 1978b).

The GC runs from the aqueous phase (Figures 3C and 3D) show the presence of oxygenated products which are soluble in water. Among the water-soluble products, 2-formylbiphenyl-2'-carboxylic acid, diphenic acid, and 9-phenantrol were found in the acidic fraction (Figure 3D). They were separated and detected as corresponding methylated derivatives by methylation prior to analysis, while other neutral water soluble products were extracted by dichloromethane prior to acidification (Figure 3C).

Under identical conditions, various suspected intermediates such as 9,10-epoxy-9,10-dihydrophenanthrene, 9,10-phenanthrenequinone, 9-fluorenone,

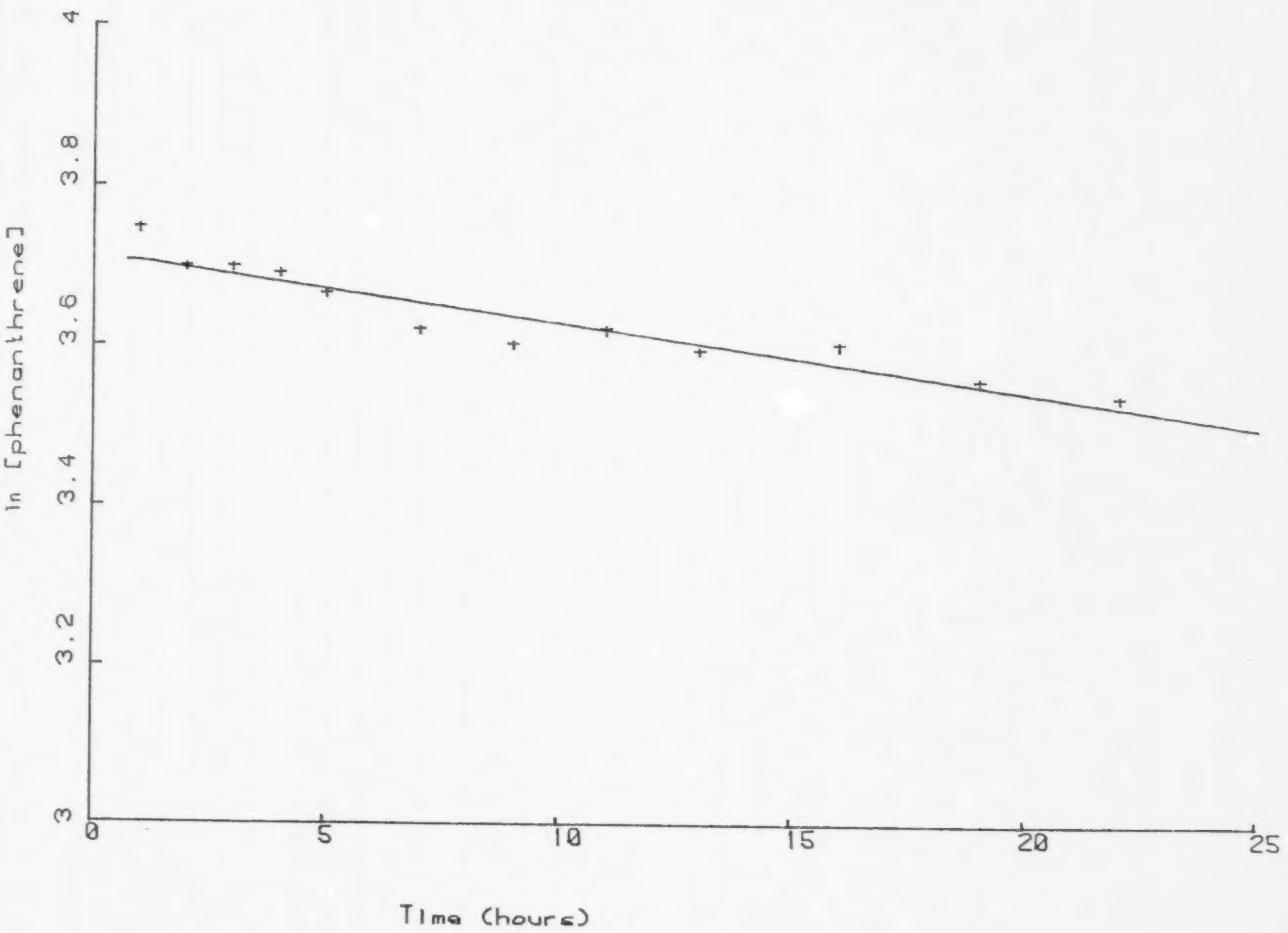

Figure 2. Kinetics of the photodecomposition of phenanthrene [natural logarithm of phenanthrene recovered (mg) x time].

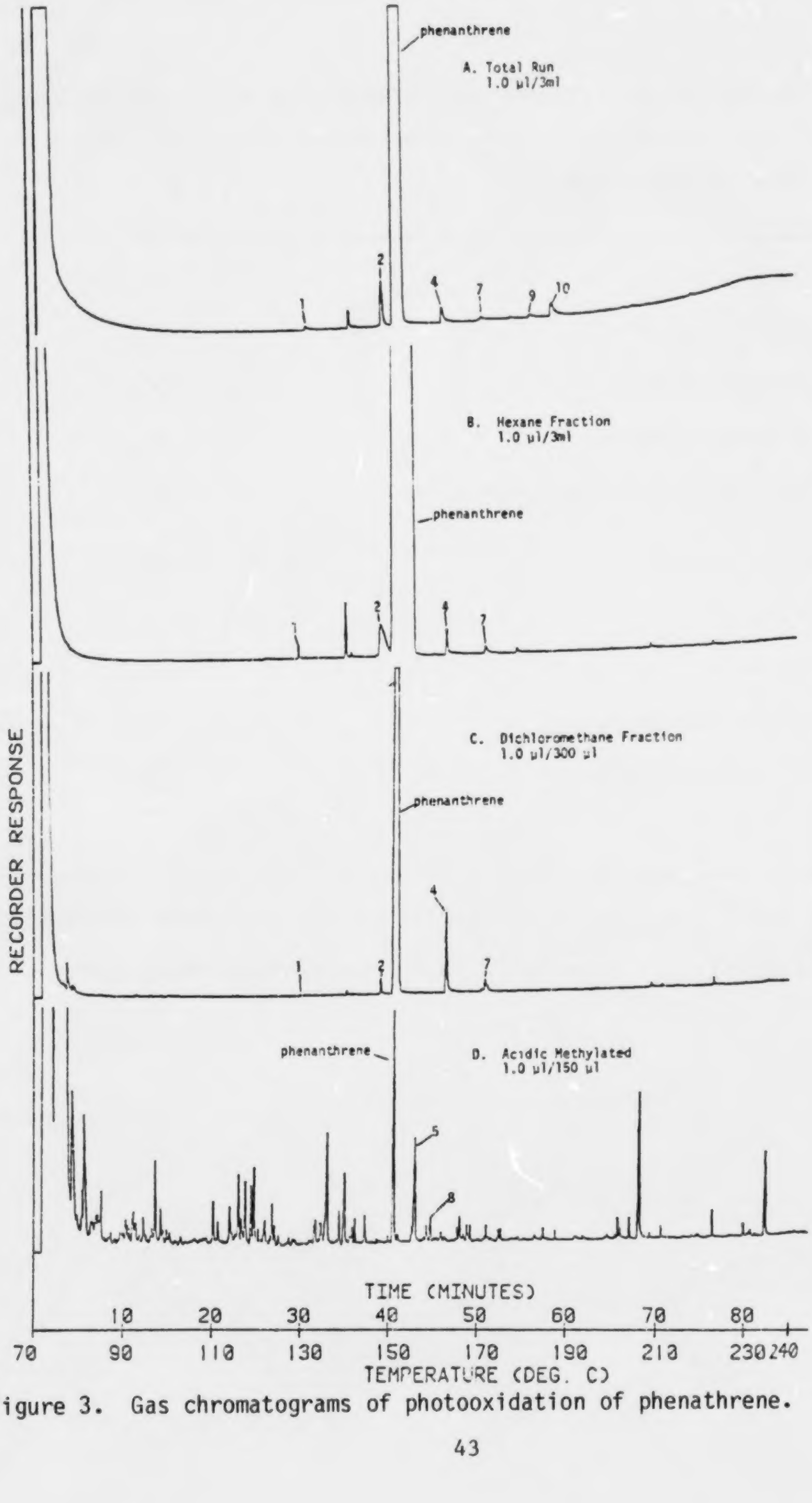

Figure 3. Gas chromatograms of photooxidation of phenathrene.

TABLE 1. PHOTOOXIDATION PRODUCTS FROM PHENANTHRENE AFTER 9 HR IRRADIATION

NO.*	NAME OF THE PRODUCTS	Formula	Mol. Wt.
1	Fluorene	$C_{13}H_{10}$	166
2	Fluorenone	$C_{13}H_8O$	180
3	2,3:4,5-dibenzoxepin	$C_{14}H_{10}O$	194
4	2,2'-diformylbiphenyl	$C_{14}H_{10}O_2$	210
** 5	2'-formylbiphenyl-2-carboxylic acid	$C_{14}H_{10}O_3$	226
7	3,4-benzocoumarin	$C_{13}H_8O_2$	196
** 8	diphenic acid	$C_{14}H_{10}O_4$	242
** 9	9-phenanthrol	$C_{14}H_{10}O$	194
10	9,10-phenanthrenequinone	$C_{14}H_8O_2$	208
** 11	diphenic acid anhydride	$C_{14}H_8O_3$	224

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* Numbers refer to the peaks in Figure 3 and the structures illustrated in Figure 4.

** These compounds were observed as their corresponding methylated derivatives.

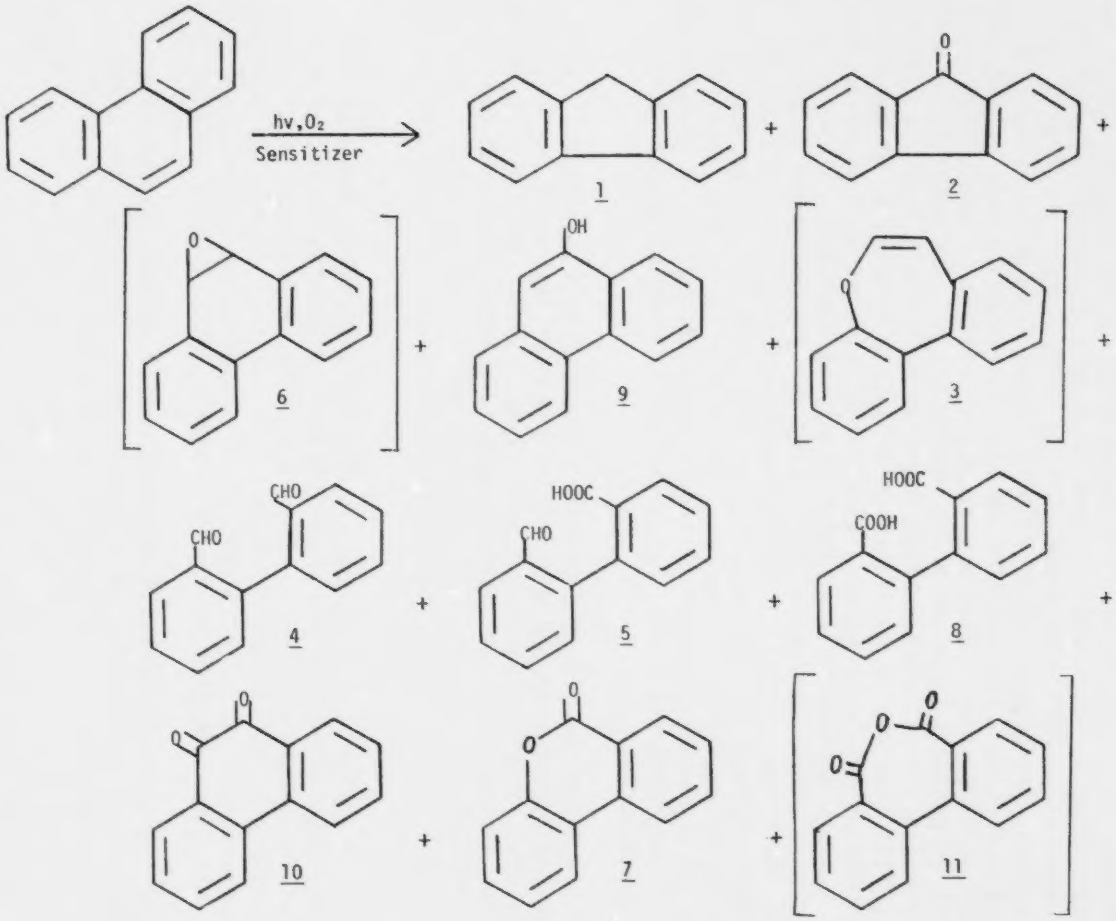

hv,O_2
Sensitizer
1
2
O
6
OH
9
3
CHO
CHO
4
HOOC
CHO
5
HOOC
COOH
8
10
7
11

Figure 4. Photooxygenated products from phenanthrene.

and fluorene were irradiated and products identified by gas chromatographic analysis. The combined gas chromatograms of the photooxidation reaction mixtures from these substrates are depicted in Figure 5.

DISCUSSION

The involvement of singlet oxygen in the photooxidation of PAHs in the environment is a very significant observation since singlet oxygen, an excited molecule, is a reactive form of oxygen responsible for various environmental oxidation (Coomber et al., 1970; Politizer et al., 1971). Furthermore, the generation of singlet oxygen from molecular oxygen using a naturally available hydrocarbon and sunlight suggests that photooxidation of various PAHs under actual environmental conditions would produce large numbers of products, some of which may be toxic. The water soluble PAHs (Frankenfeld, 1973) (phenanthrene found to be soluble in water, [May et al., 1978]) also may be subjected to oxidation by singlet oxygen available in natural waters (Zepp et al., 1977) or by singlet oxygen generated by water soluble photosensitizer and transmitted sunlight. It is known that photosensitization generally accelerates the decomposition of petroleum (Klein and Pilpel, 1974). The proposed consideration of the possible role of singlet oxygen for the oxidation of PAHs with a K-region double bond (Khan and Kasha, 1970) in chemical carcinogenesis is also justified by our study. All products identified from the photooxidation of phenanthrene seem to originate after the initial attack of singlet oxygen at the 9,10-double bond.

To achieve the actual complete conversion of phenanthrene to its oxygenated products on the air-sea interface of the ocean may require exposure to bright sunlight and sufficient oxygen. However, it is shown that the photodegradation rate constant for highly condensed PAHs increases with temperatures over the range of 5 to 31° C (Suess, 1971). Thus, at ambient temperatures, the generation of oxygenated products may be enhanced many fold.

As an integral part of the product identification phase of our program, we have studied the mass spectral fragmentation patterns of various oxygenated compounds. The structural rearrangements of several 2,2'-disubstituted biphenyls induced upon electron impact and the mass spectral comparrison of 9,10-epoxy-9,10-dihydrophenanthrene and certain other structural isomers were investigated (Patel et al., 1978b).

During the preliminary studies conducted in our laboratory on dye-sensitized photooxidation of phenanthrene (Dowty et al., 1974), complete resolution of the reaction was achieved after the proper analytical techniques were developed. We were successful in achieving our goal by using modified high resolution gas chromatography. The constituents derived from phenanthrene upon oxidation with singlet oxygen include fluorene, fluorenone, 2,2'-diformylbiphenyl, 3,4-benzocoumarin, 9,10-phenanthrenequinone, 9-phenanthrol, 2-formylbiphenyl-2'-carboxylic acid, and diphenic acid. The GC peak due to phenanthrene is off-scale and

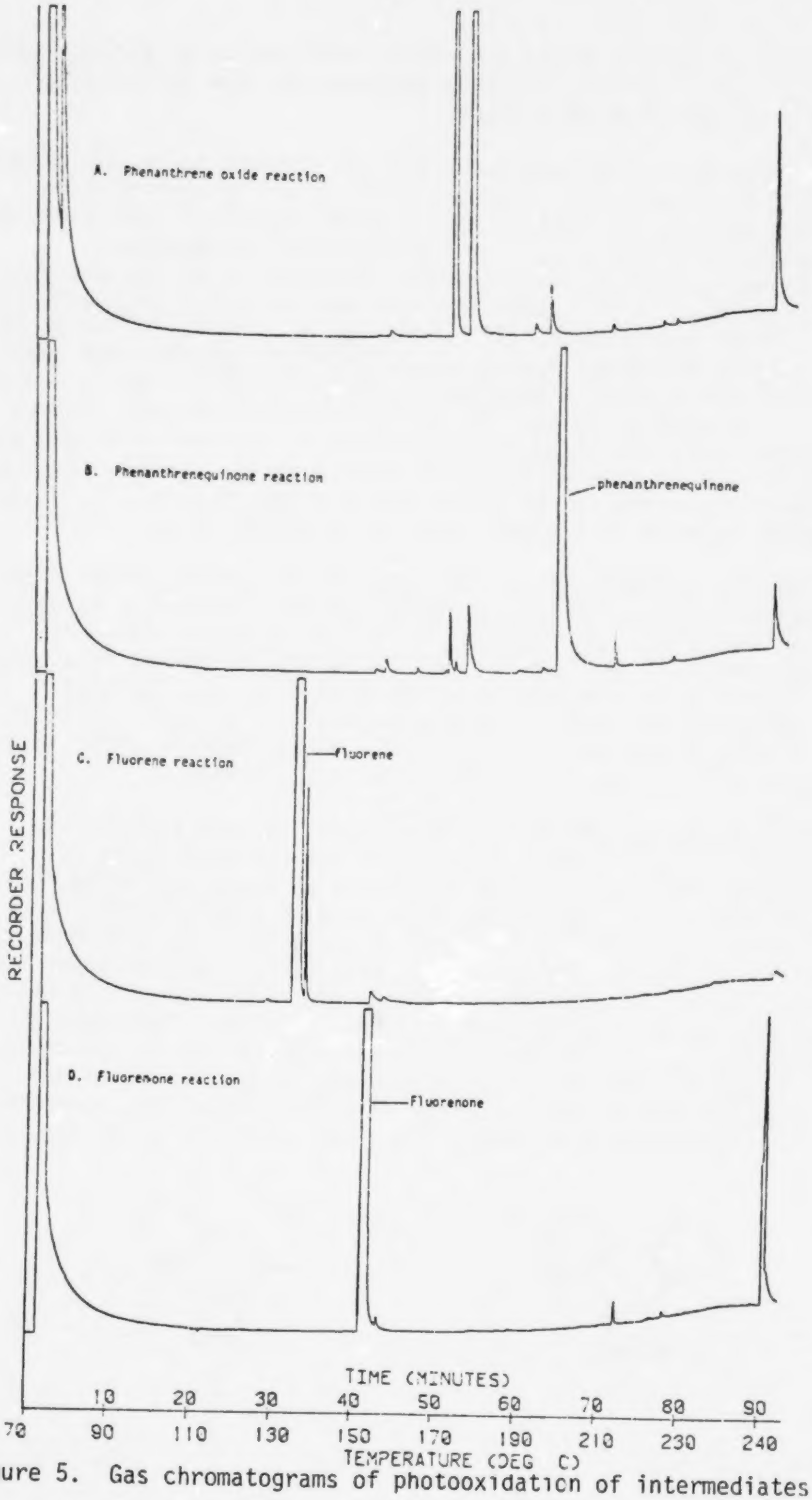


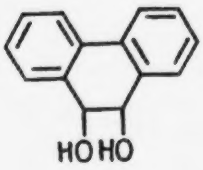
Figure 5. Gas chromatograms of photooxidation of intermediates.

unfortunately obscures a broad and significant region of the chromatogram (Figure 3). Figure 6 shows possible pathways for the formation of phenanthrene photooxidation products.

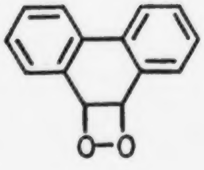
9,10-Epoxy-9,10-dihydrophenanthrene was thought to be one of the precursors for certain other photooxidation products derived from phenanthrene because its photooxidation under identical conditions gave several identical products such as 9-phenanthrol, fluorenone, 3,4-benzocoumarin, 2,3:4,5-dibenzoxepin, diphenic acid, and phenanthrene (Figures 5 and 6). It is noteworthy that during direct photooxidation reactions (Shudo and Okamoto, 1973; Dowty *et al*., 1974; Jerina *et al*., 1974) in organic solvents, 9,10-epoxy-9,10-dihydrophenanthrene gave mainly 9-phenanthrol and 2,3:4,5-dibenzoxepin, while solvolysis was a competing reaction in the aqueous phase. At pH 8.2 phenanthreneoxide converts to 9-phenanthrol (20%) and *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene (80%) due to solvolysis in aqueous solution (Bruice *et al*., 1976). Moreover, the conversion of the oxide to two other products had occurred in the gas chromatograph because of thermal sensitivity (Patel *et al*., 1978a).

Despite these complexities, the presence of 9-phenanthrol from the photooxidation products of phenanthrene, and the comparable results from the independent photolysis reaction of the oxide under identical conditions, led us to believe that the oxide may be formed as a primary product. Goto and co-workers (1978) reported that the radiolysis of liquid CO_2 with phenanthrene leads to several oxygenated products including 9,10-epoxy-9,10-dihydrophenanthrene (35%), 9-phenanthrol (46%), 2,3:4,5-dibenzoxepin (1.3%), and 2,2'-diformylbiphenyl (0.9%).

9,10-Phenanthrenequinone may have formed through a dioxetane intermediate *a* which is known to occur in the photooxidation of 9,10-dimethoxyphenanthrene or the solvoysis of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene *b*. The ready conversion of 9,10-phenanthrenequinone to several other products: fluorenone, 3,4-benzocoumarin, 2-formylbiphenyl-2'-carboxylic acid, and diphenic acid, upon photooxidation under identical conditions, may explain the occurrence of minor amounts of the quinone from the photoreaction of phenanthrene (Figures 3 and 6). Moreover, to understand the formation of fluorene as a minor product during the photoisomerization (Brightwell and Griffin, 1973; Dowty *et al*., 1974) of the oxide and to gain some insight on the stability of 9-fluorenone, the photoreactions of these two compounds have been tried under identical conditions (Figure 5).



b



a

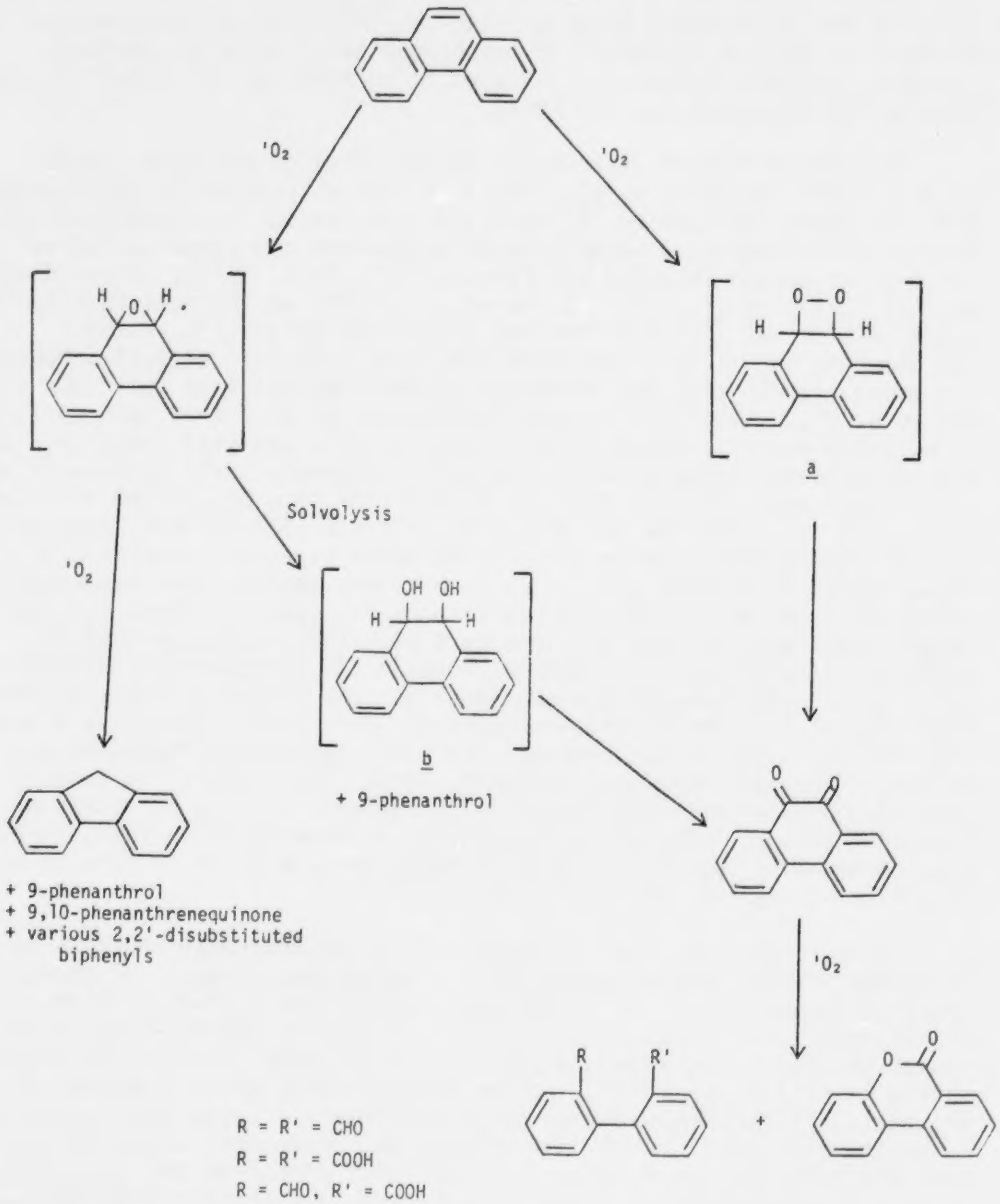

'O₂
'O₂
H O H
O — O
H H
a
Solvolysis
'O₂
OH OH
H H
b
+ 9-phenanthrol
O O
+ 9-phenanthrol
+ 9,10-phenanthrenequinone
+ various 2,2'-disubstituted biphenyls
'O₂
R R'
O O
+
R = R' = CHO
R = R' = COOH
R = CHO, R' = COOH

Figure 6. Proposed pathways for the formation of phenanthrene photooxidation products.

Most of the oxygenated products are found to be completely or partially soluble in water (Figure 3). These compounds may have great impact on marine organisms. Thus the occurrence of acidic and phenolic compounds (Guard *et al.*, 1975; Winters *et al.*, 1976; McFall *et al.*,1978) and aromatic aldehydes and ketones (Guard *et al.*, 1975; Winters and Parker, 1977) in the environment suggests that the oxidation of hydrocarbons is responsible for the formation of oxygenated water soluble compounds. Moreover, phenolic compounds have been found after an oil spill in water extracts (Burwood and Speers, 1974).

The photooxidation of aromatic hydrocarbons by sunlight (Calder *et al.*, 1978; Freegarde *et al.*, 1971), a natural process by which crude oils are decomposed during oil spills on the ocean, is a relatively slow process which generates water soluble oxygenated compounds including carbonyl compounds (Burwood and Speers, 1974; Reed, 1977). Very recent studies (Epler *et al.*, 1978; Payne *et al.*, 1978) on the mutagenicity of crude oils reveal that the fraction containing polycyclic aromatic hydrocarbons is mainly responsible for total mutagenic activity; however, the mutagenic activity was increased after irradiation of the crude oils (Payne *et al.*, 1978). It is very interesting to know that such an increase in activity was attributed to the formation of oxygenated products. In similar studies, water soluble oxygenated compounds toxic to Baker's yeast were formed upon the environmental irradiation of a No. 2 fuel oil (Larson *et al.*, 1977). Even the phytotoxicity of crude oils (Kuwait crude oil) was also increased about two to three times after exposure to artificial illumination (Lacaze *et al.*, 1977), and it was thought that oxygenated compounds after photooxidation are responsible for such changes. Thus the facts accumulated to date are in accord with the conclusion that the photooxidation of petroleum hydrocarbons in the environment increases the toxicity of petroleum and may present a serious threat to human health and catastrophic effects on marine organisms. Our studies involving a single PAH indicate clearly that carbonyl and other oxygenated compounds are generated upon environmental photooxidation. The toxicity of some of the products obtained from this study is summarized in Table 2. Moreover, compounds analogous to 3,4-benzocoumarin, 9-phenanthrol, 9,10-epoxy-9,10-dihydrophenanthrene, and diols of phenanthrene are found to have an adverse effect on biological systems (Acros, 1978).

It is significant that 9,10-epoxy-9,10-dihydrophenanthrene is believed to be the primary product among the photooxidation products of phenanthrene. A comparison of the metabolites formed from 9,10-epoxy-9,10-dihydrophenanthrene with those formed at the 9,10-bond of phenanthrene in rats showed that both compounds yielded *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (Jerena *et al.*, 1974). Both compounds also yield a mercapturic acid, N-acetyl-S-(9,10-dihydro-9-hydroxy-10-phenanthryl) cysteine. Almost all investigations of the metabolism of polycyclic aromatic compounds conclude that the formation of an epoxide is the first step from the parent compound (Sims and Grover, 1974). Arene oxides are primary metabolites of PAHs (Levin *et al.*, 1976), and many are carcinogenic in mammalian cells. They exhibit significant mutagenic (McCann *et al.*, 1975), carcinogenic (Levin *et al.*, 1976), anti-viral (Hsu *et al.*, 1977), and cellular transformation

(Marquardt et al., 1972) activity coupled with the ability to bind covalently to nucleic acids (Baird et al., 1975; Blobstein et al., 1975). The identification of an arene oxide by the environmental photooxidation of polycylic aromatic hydrocarbons is a highly significant observation for the linkage of environmental processes to carcinogenicity or mutagenicity.

TABLE 2. TOXIC PHENANTHRENE PHOTOOXYGENATION PRODUCTS*

COMPOUND	BIOLOGICAL ACTIVITY	TEST ANIMAL	DOSE	DURATION
9,10-Phenanthrene -quinone	CAR	Mouse (skin)	2000 mg/kg	28 weeks (continuous)
	TOX	Mouse (intraperit-oneal)	165 mg/kg	single dose
9-Fluorenone	CAR	Rat (subcutaneous)	360 mg/kg	26 weeks (intermitant)

*Christensen et al., 1977

CAR = Carcinogenic

TOX = Toxic

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THE MONITORING OF SUBSTANCES IN MARINE WATERS FOR GENETIC ACTIVITY

by

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ABSTRACT

Extracts of tissue from the edible mussel, Mytilus edulis, were screened for mutagenic activity using Salmonella typhimurium and a variety of strains of Escherichia coli. Genetically active material was found in the mussel extract from six of eight sites sampled in the United Kingdom. An assay of the individual tissue types from mussels at the Mumbles, Wales, site indicated that the mantle tissue was the major site of the genetically active chemical. This chemical was provisionally identified as di-2-ethylhexyl phthalate and was shown to induce mutation predominantly by insertion or deletion of nucleotide bases.

INTRODUCTION

The use of the marine environment as a sink for the disposal of chemicals both deliberately and accidently suggests that at least a fraction of the living organisms found in the seas and oceans may be exposed to potentially mutagenic agents. Such exposure may result in changes in the genetic architecture of marine populations; if such agents enter food chains, they may lead to the unwitting exposure of human populations.

A variety of screening systems have been developed for the use of biological indicator organisms in the detection of the possible mutagenic activity of environmental chemicals. Such systems involve the use of organisms varying from relatively simple bacteria to mammalian cells in tissue culture. The systems utilize the procedure of exposing cells of known genotype to test agents and the plating of treated cells upon selective medium to detect those cells which have been mutated by the agent. In a large number of cases, mutagenic activity in the screening systems has been shown to be correlated with the ability of a chemical to produce tumours either in experimental animals or humans (McCann _et al_. 1975).

The assay of the seas and oceans for the presence of mutagenic chemicals can be performed in two fundamentally different ways. Chemical analysis can be performed with ocean samples to identify constituent chemicals, which can be individually tested for mutagenic activity. This is a formidable task because of the very large number of chemicals both natural and man-made that are detectable and must therefore be screened for mutagenic activity. However, such studies performed on the constituents of some samples of drinking water in the U.S. have revealed the presence of mutagenic chemicals (Simmons _et al_., 1977).

The second approach, involves the collection of ocean samples, concentration of the constituents, and the exposure of the resulting concentrate to a range of mutagenic screening systems. However, this procedure has at least two fundamental limitations: the problem of developing a suitable concentration procedure and, perhaps more importantly, the difficulty of detecting mutagens in the presence of toxic chemicals. For example, a mutagenic chemical might be undetectable if the assay is performed in the presence of a chemical which is highly toxic to the test organism.

We attempted to overcome the problems of the latter procedure by using marine organisms as concentrators of potentially mutagenic chemicals. By the use of such living organisms, we have also selected against the possible masking effects of toxic chemicals on the assumption that such toxicity would lead to the death of the marine species in question. We have developed an assay system based upon the extraction of tissues derived from the mussel, _Mytilus edulis_, and the screening of these extracts for mutagenic activity, using a number of microbial indicator species. We were able to identify the presence of an industrial chemical capable of inducing mutation in bacteria.

MATERIALS AND METHODS

Strains

The strains of bacteria used in these studies included cultures of _Salmonella typhimurium_, auxotrophic for histidine (kindly provided by Dr. Bruce Ames) and a variety of _Escherichia coli_ strains (kindly provided by Dr. G. Mohn, Dr. M. Green, and Dr. D. Tweats or our laboratory). They have been described in the literature (Parry _et al_., 1976) and are capable of detecting both frameshift and base-substitution mutagens.

The yeast strain *JD1* was auxotrophic for both histidine and tryptophan and produced prototrophic colinies by the process of mitotic gene conversion, a process of genetic change that responds to treatment by mutagens and carcinogens in an essentially non-specific manner. The use of this yeast strain has been described in detail elsewhere (Davies *et al*., 1975).

Preparation of Mussel Extracts

Samples of the mussel, *Mytilus edulis*, were collected from a variety of sites, washed with running water and either extracted immediately or kept frozen at -20° C until required. (There was no evidence in our work of any reduction in genetic activity during storage.)

For the preparation of extracts of whole mussel tissue, 50 shelled mussels were extracted in 100 mℓs of 95% ethanol after disintegration of the tissue in a Waring blender or Atomix. The resulting tissue homogenate was left to stand overnight at 4° C, resuspended and centrifuged at 5000 g for 15 min. The supernatant was sterilized by passing through a membrane filter and stored at 4° C for use within one week and at -20° C for long-term use. All the samples obtained were tested for the presence of radioactive material. None of the samples described here showed levels of radioactivity above the background.

Detection of Genetic Activity

Fluctuation tests were carried out, with minor modifications, as described by Green *et al*. (1976). Overnight cultures of *E. coli* and *S. tryphimurium* tester strains were prepared in supplemented Davis-Mingioli minimal medium. After two rinses in saline, the cells were resuspended in saline at a concentration of 5.0×10^7 cells per mℓ; 100 mℓ Davis-Mingioli basal salts were combined with 0.7 mℓ 40% glucose, 0.1 mℓ tryptophan solution (200 μg/mℓ) (for fluctuation tests involving tryptophan auxotrophs only), or 0.1 mℓ lysine solution (200 μg/mℓ) (for lysine auxotrophs only), or 0.1 mℓ of histidine solution (200 mg/mℓ) (for histidine auxotrophs only), 0.1 mℓ washed cells, and, in the case of the test treatments, either methyl methane sulphonate (final concentration, 1 μg/mℓ) as a positive control, or 0.1 mℓ of alcoholic mussel extract. For experiments involving the use of multiple auxotrophs, the supplements required by the non-selected markers were added in excess. Control experiments involving mussel extracts also contained 0.1 mℓ 95% ethanol as a negative control. Each treatment was dispensed in mℓ samples into 50 test tubes or in some cases 1 mℓ samples into 100 test tubes. After the auxotrophic bacteria exhausted the small amount of supplement present, only prototrophic revertants continue to grow. From 2 days and thereafter, tubes in which mutation had occurred became turbid, while other tubes remained relatively clear. The number of turbid tubes was routinely scored after 3 days. The significance of the response of each set of 50 tubes to the presence of mussel extract was determined by the use of Chi-square analysis as described by Green *et al*. (1976). The arginine-56 mutation used in some experiments is leaky, and as a result some residual growth was observed even in the absence of arginine. Therefore, trace amounts of arginine were not added when this marker was used.

In a number of experiments, the fluctuation test was modified according to the procedure developed by Dr. David Gatehouse (personal communication): samples were added to 96-well microtitre plates (Sterilin, Ltd.) in the form of 0.2 mℓ aliquots per well. All other steps in the procedure were as described above.

In the yeast assays, we used stationary phase cultures of the strain *JD1* suspended in saline at a concentration of 10^7 cells/mℓ. Samples of the mussel extracts (up to 4%) were added to the yeast minimal medium at 45°C together with yeast cells at a concentration of 35×10^3 cells/mℓ for the detection of histidine-independent prototrophs and 35×10^2 cells/mℓ for the detection of tryptophan-independent prototrophs and poured into 9-cm Petri dishes. The culture medium was supplemented with 20 µg/mℓ tryptophan and 0.1 µg/mℓ histidine for the detection of histidine-independent prototrophs and with 20 µg/mℓ histidine and 0.1 µg/mℓ tryptophan for the detection of tryptophan-independent prototrophs. These supplements enabled the auxotrophic cells to undergo three cell divisions in the presence or absence of mussel extracts. All plates were grown in the dark at 28° C and scored after 9 days of incubation. In all experiments involving the yeast cultures, we used at least five replicate plates per treatment.

CHEMICAL ANALYSIS OF MUSSEL EXTRACTS

Fifty-gram samples of mussel mantle tissue were homogenized in 1 ℓ 2:1 chloroform/methanol in a blender. The homogenized material was left overnight and then filtered and treated with 200 mℓ 0.05M KCl. The resulting organic layer was dried with 50 gm of sodium sulphate and filtered; the whole extract was evaporated to dryness in a rotary evaporator. The residual material was taken up in 5 mℓ pentane and applied to a 200 mℓ silica-gel column. Solvents then were run through the column in the following order: 1. pentane, 2. 95% pentane + 5% ether, and 3. chloroform. The individual ellutants were collected and rotary dried; the residual material from each sample was taken up in either ethanol or DMSO and tested for mutagenicity.

After the major fraction of the detectable mutagenic activity was verified in the chloroform extract, the sample was separated further. A portion of the chloroform extract was applied to a preparative thin layer chromatography plate. The plate was then run in one dimension, using chloroform. After the solvent front reached the end of the plate, the plate was dried and examined. Of the 9 labelled zones, 3, 6, and 8 were fluorescent when observed in UV light. Each zone was scraped from the plate and shaken up with 95% ethanol; the sample obtained was filtered through a membrane filter and tested individually for mutagenicity. All samples were coded by a colleague and tested blind.

RESULTS

Geographic Variation in the Presence of Genetically Active Chemicals

Alcoholic extracts of the whole tissue of mussels collected from a variety of geographical locations were screened for the presence of genetically active chemicals by the measurement of cells prototrophic for histidine and tryptophan produced by induced mitotic gene conversion in the yeast, Saccharomyces cerevisiae. The sites sampled were Swansea, Mumbles, Caswell Bay, Solva, Llangranog, and Milford Haven in South Wales, Anglesey in North Wales, and Plymouth in Southwest England. The samples were screened in two experimental series as shown in Table 1, using ethanol as a negative control and the alkylating agent ethyl methane sulphonate, as a positive control.

The results (Table 1) demonstrate the presence of genetically active material in the samples collected from Plymouth, Caswell Bay, Mumbles, Swansea, Milford Haven, and Llangranog; no such activity was detectable in samples collected from Anglesey and Solva.

Further confirmation of the site variation in genetic activity was obtained by examining alcoholic extracts of mussels collected on the same day at a series of sites westward from Mumbles. These samples were screened for genetic activity by the measurement of mitotic gene conversion, using the yeast strain JD1. The results obtained from these assays (Table 2) demonstrate that genetic activity decreased in the samples collected at increasing distances from the Mumbles site. The decreased genetic activity correlates with reduced visual pollution and increased distance from the industrial area surrounding Swansea Bay.

Tests for the Presence of Nutrients in the Mussel Extracts

The majority of the tests to detect genetic activity in mussel tissues are based upon the measurement of the production of prototrophic colonies in auxotrophic microbial cultures. Assays of this type may lead to inconclusive results if the samples contain significant amounts of the specific nutrient deleted from the selective media. In such cases, additional growth of the test culture would lead to a spurious positive result. Thus, it was necessary for us to ensure that all the samples did not contain significant amounts of nutrients.

In the case of the bacterial fluctuation tests, superficial estimates of the presence of nutrients can be made by visual and colorimetric examination of the clear tubes, i.e., those that show only background growth due to the presence of the original auxotrophic cells. When significant amounts of nutrients were present in the mussel extracts, increased background growth in the tubes was readily observable. If free nutrients were present, the samples were rejected for assay, or used in a mutagenicity assay that did not require the contaminating nutrient was used.

TABLE 1. THE INDUCTION OF MITOTIC GENE CONVERSION IN YEAST IN THE PRESENCE OF A VARIETY OF MUSSEL EXTRACTS

Treatment	Mean no. of his$^+$ prototrophs per plate	his$^+$ prototrophs per 10^6 survivors ±s.e.	Mean no. of trp$^+$ prototrophs per plate	trp$^+$ prototrophs per 10^5 survivors ±s.e.	% Viability
Experimental series 1 Control 1	8.6	26.8 ± 4.6	7.4	23.1 ± 4.3	91.4
Control 2	7.8	32.5 ± 5.8	5.2	21.7 ± 4.8	68.6
4% Alcohol control 1 Negative Controls	6.5	25.6 ± 5.0	9.4	37.0 ± 6.0	73.1
4% Alcohol control 2	4.2	11.8 ± 3.0	7.6	21.4 ± 3.9	101.4
Plymouth extract 4%	202.6	493.6 ±21.3	154.8	463.5 ± 18.6	95.4
Mumbles extract 4%	71.2	323.6 ±19.2	85.3	387.7 ± 20.6	62.9
Caswell Bay 4% extract	69.0	276.0 ±16.6	69.2	276.8 ± 16.6	71.4
Anglesey 4% extract	4.7	18.3 ± 4.2	7.6	29.6 ± 5.4	73.4
Ethyl methane sulphonate (1μg/mℓ) - Positive Control	284.7	900.9 ±26.7	346.2	1,095.0 ± 29.4	90.3
Experimental series 2 Control 1	21.4	62.2 ± 8.4	11.4	33.1 ± 5.1	98.3
Control 2	28.7	84.9 ± 9.7	9.3	27.6 ± 4.8	96.5
Swansea (St. Helens) 4%	91.0	256.7 ±12.3	68.2	192.6 ± 11.6	101.3
Solva (S. Wales) 4%	19.4	53.2 ± 9.1	8.7	23.9 ± 8.4	104.1
Milford Haven (S. Wales) 4%	53.6	175.4 ±12.1	61.3	200.4 ± 15.6	87.3
Llangranog (S. Wales) 4%	83.1	284.7 ±11.9	114.1	390.9 ± 19.7	83.4
4% Alcohol control 1	17.3	65.4 ± 8.4	5.9	22.3 ± 4.3	75.6
4% Alcohol control 2	15.9	47.3 ± 7.1	7.2	21.1 ± 3.1	97.6
Ethyl methanesulphonate (1μg/mℓ)	317.2	1,050.7 ±29.6	214.9	712.3 ± 27.0	86.2

TABLE 2. THE INDUCTION OF MITOTIC GENE CONVERSION IN YEAST IN THE PRESENCE OF MUSSEL EXTRACTS COLLECTED IN THE GOWER AREA OF SOUTH WALES

	his$^+$ prototrophs per 10^6 survivors ±s.e.	trp$^+$ prototrophs per 10^5 survivors ±s.e.	% cell viability
Control 1 4% ethanol	19.7 ± 1.9	7.1 ± 1.2	84.2
Control 2 4% ethanol	24.2 ± 2.2	8.9 ± 1.3	73.3
Mumbles 4%	621.4 ± 11.1	247.5 ± 7.0	65.4
Caswell Bay 4%	492.9 ± 7.0	132.0 ± 3.6	78.5
Oxwich 4%	34.2 ± 1.8	15.1 ± 1.2	82.2
Port Eynon 4%	29.0 ± 1.7	9.2 ± 0.9	74.0
Rhossilli 4%	31.3 ± 2.5	6.4 ± 1.1	68.6

Samples that showed positive genetic activity were further investigated for the presence of free nutrients by making viable counts of the numbers of bacterial cells present in both the clear and turbid tubes in the fluctuation tests. A typical example of the results of such an assay (Table 3) shows the viable cell counts made from individual fluctuation test tubes, using the *Escherichia coli* culture uvrAtrp(R46) in both the presence and absence of Mumbles mussel extract. The results demonstrate that the median and mean numbers of viable bacterial cells per clear tube, that is, in tubes that do not contain a significant number of trp$^+$ revertants, were unaffected by the presence of mussel extracts. Therefore, we can conclude that the particular sample of Mumbles mussel extract did not enhance the growth of trp-cells when the added tryptophan supply had become exhausted. We can further conclude that the observed increase in the frequency of turbid tubes in the bacterial fluctuation test was not due to increased cell division in the presence of free nutrients, which would have allowed the production of an increased frequency of spontaneous mutants.

The possible nutrient effects of the mussel extracts upon auxotrophic yeast cultures were determined in two ways:

1. After the incubation period, the plates of selective agar were scored for the frequency of prototrophic colonies and then discs of agar 9 mm in diameter were removed from areas of the plates showing background growth, but not large prototrophic colonies. Agar discs sampled from 10 plates per treatment were added to 20 mℓ sterile saline; yeast cells contained in the discs were suspended by sonication. After appropriate dilutions, the saline suspensions were plated upon yeast complete agar, and the colonies produced were counted after 5 days incubation at 28°. Comparisons of the viable cells contained in the background agar of both the treated and untreated cultures then could be used to determine if any further growth of auxotrophic yeast cells had taken place in the solid medium in the presence of the mussel extract. The results of such an assay using the mussel extract derived from the Plymouth site (Table 4) demonstrate that there are no significant differences in the growth of auxotrophic cells between the plates with and without mussel extract.

2. Samples of the mussel extracts were added to liquid minimal medium containing either excess histidine or excess tryptophan together with 10^5 yeast cells/mℓ of strain JD1 auxotrophic for both histidine and tryptophan and the cultures were aerated for periods of up to 48 hr. During this period cell growth was determined by counting cell numbers with a heamocytometer. Under these conditions, samples that contain nutrients showed increases in cell numbers during the first 6 hr incubation. In samples lacking nutrients, no increases in cell numbers were observed until after approximately 24 hr when the growth of pre-existing prototrophic cells become significant. The results of a typical experiment of this type are shown in Figure 1.

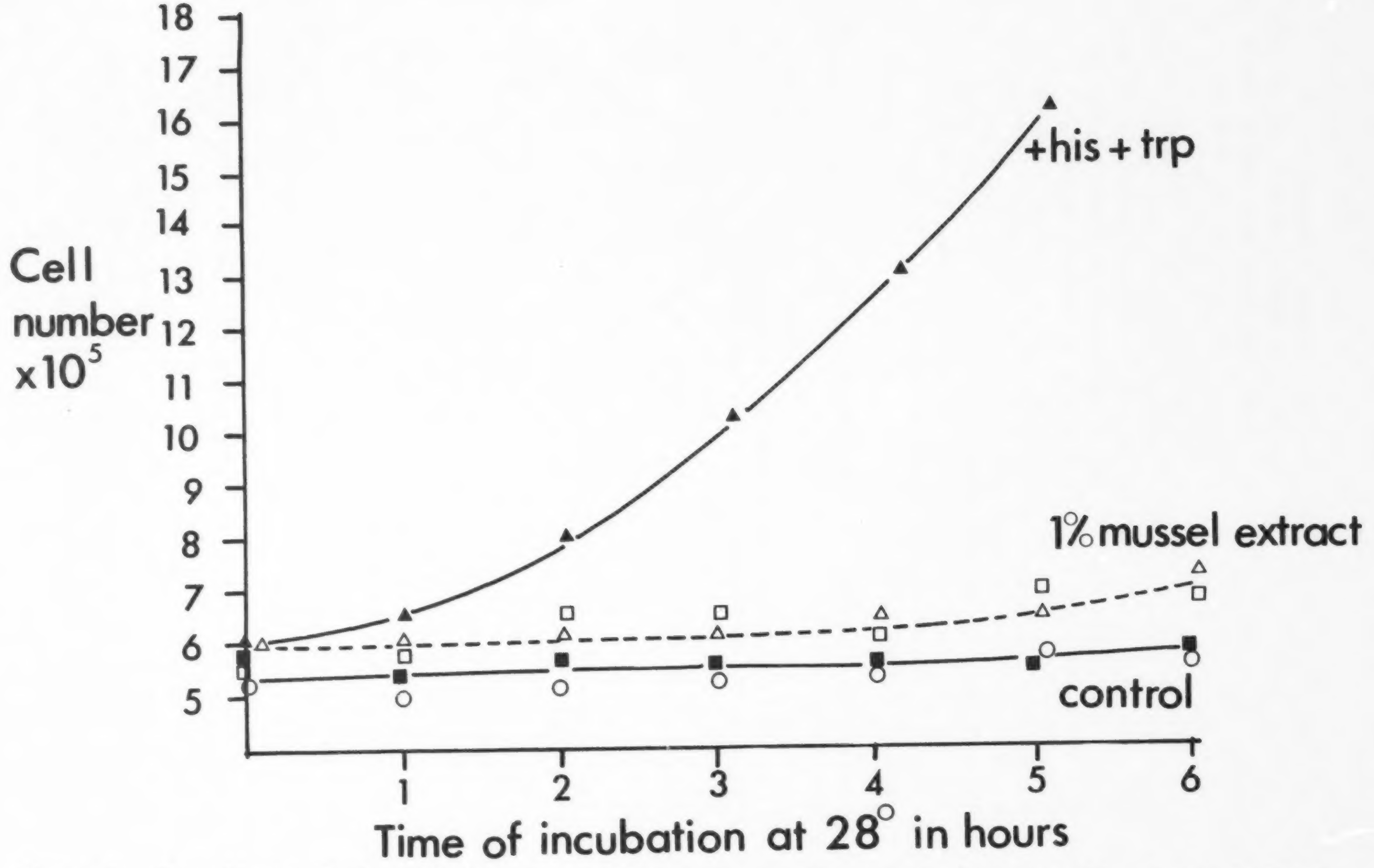

Figure 1. The effects of the addition of mussel extract upon the growth of yeast cells of strain JDI in Yeast Minimal Medium; ▲ -medium supplemented with 20 µg/mℓ histidine and tryptophan; ○ -medium supplemented with 20 µg/mℓ histidine only; ■ -medium supplemented with 20 µg/mℓ histidine and 1% ethanol; □ -medium supplemented with 20 µg/mℓ histidine and 1% mussel extract; △ -medium supplemented with 20 µg/mℓ trytophan and 1% mussel extract.

TABLE 3. VIABLE CELL COUNTS OF TURBID AND CLEAR TUBES FROM A FLUCTUATION TEST INVOLVING THE USE OF *ESCHERICHIA COLI* CULTURE UVR A TRP (R46) WITH AND WITHOUT THE ADDITION OF MUSSEL TISSUE EXTRACT FROM THE MUMBLES AREA

	Mean value turbid tubes cells/mℓ	Mean value clear tubes cells/mℓ	Median value clear tubes cells/mℓ
Control	5.9×10^8	5.3×10^6	1.27×10^7
Control + 0.1 mℓ ethanol	6.0×10^8	5.0×10^6	1.95×10^7
0.1 mℓ Mussel extract	5.3×10^8	5.6×10^6	9.0×10^6

TABLE 4. ASSAY OF THE BACKGROUND GROWTH OF YEAST CELLS FROM THE PLATES OF SELECTIVE AGAR WITH AND WITHOUT THE PRESENCE OF ADDED MUSSEL EXTRACT (PLYMOUTH)

Treatment	Background growth, mean of discs removed from selective plates used for the detection of his$^+$ prototrophs cells/mℓ	Background growth, mean of discs removed from selective plates used for the detection of trp$^+$ prototrophs cells/mℓ
Control	$2.9 \pm 0.5 \times 10^5$	$4.1 \pm 0.8 \times 10^4$
Control + 1% ethanol	$3.2 \pm 1.1 \times 10^5$	$3.7 \pm 1.2 \times 10^4$
1% Mussel extract	$2.4 \pm 0.7 \times 10^5$	$3.5 \pm 1.1 \times 10^4$
2% Mussel extract	$1.9 \pm 0.8 \times 10^5$	$4.3 \pm 0.8 \times 10^4$
4% Mussel extract	$1.7 \pm 0.4 \times 10^5$	$4.0 \pm 1.3 \times 10^4$

None of the mussel samples reported here to be mutagenic were demonstrated to contain significant amounts of the specific nutrient required by the test microbes. However, we detected such nutrients in many samples; in particular we found that extracts of fish tissue also studied in our laboratory contained significant amounts of free amino acids, particularly histidine.

The Assay of Specific Mussel Tissues

The experiments described above demonstrate the presence of genetically active chemicals in alcoholic extracts derived from all the tissues of the organism. We also determined whether this activity was distributed throughout all the tissues of the organism or concentrated within specific tissues.

Initially we separated the body tissues into (1) the hepatopancreas and (2) all the remaining tissues; 10 gm of sample (1) and sample (2) were then extracted in 50 mℓ 95% ethanol. These extracts of hepatopancreas and the remaining tissues were screened for genetic activity with a range of bacterial strains in a series of fluctuation tests. The results of these fluctuation tests (Table 4) demonstrate that mutagenic activity was not detectable in the hepatopancreas but was found in the remaining tissue of mussels from the Mumbles site.

Further subdivisions of the mussel tissue were made and extracts prepared from mantle, foot, muscle, and the reproductive system at a concentration of 10 gm of tissue in 50 mℓ 95% ethanol. Samples of each of the tissue extracts were assayed for the presence of genetically active chemicals by the measurement of induced mutation in a series of fluctuation tests and by the measurement of induced mitotic gene conversion in yeast.

The results of the assays of the various mussel tissues for genetic activity are shown in Table 5 (bacterial tests) and Table 6 (yeast tests). Both the mutation tests with bacteria and those of induced mitotic gene conversion in yeast demonstrate that the major site of genetically active chemicals in mussels collected from the Mumbles site was the mantle tissue.

Identification of the Molecular Nature of Mutagenic Present in the Mussel Extract

Mutagenic chemicals may be classified on the basis of the type of change that they produce in cellular DNA. The strains of bacteria used in this work made it possible to determine whether a mutagenic chemical produces predominantly base change mutations leading either to missense or nonsense changes at the level of protein synthesis or to the insertion or deletion of nucleotide bases leading to a change in the reading frame of at the level of protein synthesis (frameshift).

TABLE 5. EFFECTS OF MUSSEL EXTRACTS FROM A VARIETY OF DIFFERENT TISSUES UPON INDUCED MUTATION, MEASURED IN BACTERIAL FLUCTUATION TESTS USING ESCHERICHIA COLI

Strain	Locus	Treatment	No. of tubes sampled	No. of tubes positive		Significance (probability)
				Control	Treated	
341/113	arg 56	MMS	50	24	41	< .01
		Hepatopancreas	50	24	34	NS
		Remaining tissue	50	24	41	< .01
341/113 (R46)	arg 56	MMS	50	28	48	< .001
		Hepatopancreas	50	35	41	NS
		Remaining tissue	50	35	45	< .01
uvr A (R46)	trp	MMS	100	54	87	< .001
		Hepatopancreas	100	48	59	NS
		Remaining tissue	100	48	70	< .05
343/113	arg 56	MMS	50	21	39	< .01
		Mantle	50	24	44	> .05
		Foot	50	24	34	NS
343/113 (R46)	lys 60	MMS	50	28	40	< .02
		Mantle	50	28	40	< .02
		Foot	50	28	35	NS
uvr A (R46)	trp	Mantle	50	15	28	< .02
		Foot	50	15	18	NS
		Reproductive system	50	15	18	NS

MMS = Positive control methyl methane sulphonate

NS = not significant

TABLE 6. EFFECTS OF MUSSEL EXTRACTS FROM A VARIETY OF DIFFERENT TISSUES UPON THE INDUCTION OF MITOTIC GENE CONVERSION IN YEAST STRAIN JD1

Treatment	Viability	trp^+ prototrophs per 10^5 survivors ±s.e.	his^+ prototrophs per 10^6 survivors ±s.e.
Control	100.0	19.1 ± 1.8	36.7 ± 2.3
Negative control 2% ethanol	101.3	22.4 ± 1.7	32.4 ± 1.9
Muscle 1%	97.3	16.6 ± 1.4	37.5 ± 2.3
2%	94.4	29.3 ± 1.8	41.0 ± 2.9
Mantle 1%	101.9	94.5 ± 3.9	170.3 ±11.6
2%	93.2	135.7 ± 8.2	589.6 ±23.4
Hepatopancreas 2%	85.3	27.9±+ 1.5	41.6 ± 2.7
Foot 2%	92.4	17.6 ± 0.9	39.4 ± 2.1
Reproductive system 2%	76.8	14.3 ± 2.3	20.7 ± 1.9

Table 7 summarizes a number of experiments that were performed with the mussel extract from the Mumbles site, using a variety of bacterial cultures in fluctuation tests. The cultures of Escherichia coli used in the tests have been described in Material and Methods and are capable of detecting the presence of both frameshift and base change mutagens by the induction of prototrophs at specific genetic markers.

As seen in Table 7, the extract of mantle tissue from mussels collected at Mumbles was capable of inducing prototrophs only at those loci, i.e. lys 60, arg 56, and gal R^s, and of reverting by frameshift events. It was without activity at the trp locus, which is capable of reversion by base change mutation. Thus we conclude that the particular chemical agent present in the mantle tissue of mussels collected from the Mumbles site is capable of inducing mutation predominantly by the mechanism of insertion or deletion of nucleotide bases.

Chemical analysis of the contents of mussel extracts

To determine the nature of those chemicals present in the mussel extracts which show genetic activity, we prepared the samples in 2:1 chloroform/methanol as described in Materials and Methods. After fractionation on a silica-gel column extracts made up in pentane, 95% pentane + 5% ether and chloroform were obtained and tested for mutagenic activity with the Salmonella typhimurium strain TA98 in a series of fluctuation tests.

The results obtained for pentane, pentane and ether, and chloroform extracts of all mussels from Mumbles (Table 8) demonstrate that the chlorofrom extract contains the major activity as shown by an increase in mutation at the his D locus of Salmonella strain TA98 measured in fluctuation test experiments, using three separate extractions of mussel tissue from the Mumbles site.

The chloroform extract was further separated by thin layer chromatography to produce nine separate samples labelled 1 to 9, three of which (namely 3, 6, and 8) were fluorescent when observed on a chromatography plate. Each zone was removed from the plate and shaken with 95% ethanol and the resulting extracts tested for the presence of mutagenic chemicals. The results of the mutagenicity tests of the extracts of the zones using bacterial fluctuation tests involving the Salmonella strain TA98 (Table 9) demonstrate that the zones 3, 6, and 8 contain mutagenic chemicals that produce a significant increase in the number of positive tubes in the fluctuation tests.

The residue from the UV fluorescent band 3 (Rf 0.74) was examined by proton magnetic resonance with a high sensitivity F.T. instrument (Varion XL-100). The presence of a long chain phthalate ester was stabilized and identified by its characteristic mass spectrum, as di-2-ethylhexyl phthalate, with a high resolution MS-9 mass spectrometer. The nmr and mass spectra were identical to a commercial sample of di-2-ethylhexyl phthalate. A quantitative estimation of the phthalate by nmr indicated the presence of approximatley 11 μg per mussel.

TABLE 7. SPECIFICITY OF ACTION OF ALCOHOLIC EXTRACTS OF MANTLE AND FOOT TISSUES OF MUSSELS COLLECTED FROM MUMBLES UPON MUTATION INDUCTION IN THE BACTERIAL FLUCTUATION TEST USING CULTURES OF *ESCHERICHIA COLI*

343/113 (R46)	lys 60 predominantly frameshift	Expt 1	MMS	50	28	40	<.02
			Mantle	100	42	74	<.01
			Foot	100	42	61	NS
		Expt 2	Mantle	50	28	42	<.01
			Foot	50	28	31	NS
		Expt 3	Mantle	50	16	34	<.01
			Foot	50	16	16	NS
		Expt 4	MMS	50	28	40	<.02
			Mantle	50	28	40	<.02
			Foot	50	28	33	NS
343/113	arg 56 base change and frameshift	Expt 1	MMS	50	21	39	<.01
			Mantle	50	21	34	<.02
			Foot	50	21	24	NS
		Expt 2	Mantle	50	19	30	<.05
			Foot	50	19	19	NS
343/113	gal R^S base change and frameshift	Expt 1	Mantle	75	33	51	<.01
			Foot	75	33	39	NS
WP2 uvrA	trp base change	Expt 1	MMS	50	15	32	<.001
			Mantle	100	24	32	NS
			Foot	100	24	26	NS
		Expt 2	MMS	50	15	31	<.01
			Mantle	50	15	16	NS
			Foot	50	15	16	NS
		Expt 3	Mantle	50	9	16	NS
			Foot	50	9	10	NS

MMS = positive control mutagen methyl methane sulphonate.

NS = no significant difference in the frequency of positive tubes in the control and treated series of tubes.

TABLE 8. GENETIC EFFECTS OF SOLVENT EXTRACTS AS MEASURED IN BACTERIAL FLUCTUATION TESTS USING THE STRAIN SALMONELLA TYPHIMURIUM TA 98

Strain	Location	Solvent (% solution)	No.of tubes tested	No.of tubes positive		Significance
				Control	Treated	
TA 98	Mumbles	.1 Pentane	50	10	11	NS
		.1 Pentane + ether	50	10	16	NS
		.1 Chloroform	50	10	26	< .01
TA 98	Mumbles	.1 Pentane	50	8	9	NS
		.1 Pentane + ether	50	8	7	NS
		.1 Chloroform	50	8	28	< .001
TA 98	Mumbles	.1 Pentane	50	10	13	NS
		.1 Pentane + ether	50	10	12	NS
		.1 Chloroform	50	10	23	< .01

TABLE 9. GENETIC EFFECTS OF EXTRACTS OF EACH ZONE MEASURED IN A BACTERIAL FLUCTUATION TEST USING BACTERIAL STRAIN _SALMONELLA TYPHIMURIUM_ TA 98

Strain	Zone	No.of tubes sampled	No.of tubes positive Control	Treated	Significance
TA 98					
	1	50	10	9	NS
	2	50	10	15	NS
	* 3	50	10	22	< .02
	4	50	10	13	NS
	5	50	10	12	NS
	* 6	50	10	23	< .01
	7	50	10	11	NS
	* 8	50	10	21	< .05
	9	50	10	20	NS

Zone 6 was identified as containing 2 - ethylhexyl phthalate

Because phthalate esters are known to be found in biological extracts due to the extraction of residual plasticisers in solvents, plastic utensils, and tubing, it was considered necessary to carry out a blank extraction procedure as far as the silica gel column stage with the same volumes of solvents and the same apparatus used in the mussel extraction. The residue at the end of this blank extraction procedure was shown by nmr and mass spectra to contain only negligible quantities of phthalates, thus demonstrating that the phthalate isolated from the mussels was genuinely present in the mussel tissue.

To confirm the mutagenic potential of phthalate esters, we investigated the activity of a commercial sample of phthalate esters di-iso-octyl phthalate (B.D.H. Poole, Dorset), which was known to contain high levels of di-2-ethylhexyl phthalate. The results (Table 10) obtained with a variety of strains of bacterial cultures in a series of fluctuation tests demonstrate that significant increases in mutation were detected with concentrations of 1 and 5 μg/mℓ di-iso-octyl phthalate in Salmonella strain TA98 and in some experiments with the Escherichia coli strain 343/113.

Further confirmation of the mutagenic activity of di-iso-octyl phthalate was provided by a series of fluctuation tests, using the Salmonella strain TA98 in the presence of a rat liver microsome extract (S-9 mix) and appropriate co-factors. The results (Figure 2) demonstrate that concentrations of di-iso-octyl phthalate of 2 to 4.5 μg/mℓ produce significant increases in the frequency of turbid tubes produced by mutation to prototrophy. The results also indicate that at higher concentrations of di-iso-octyl phthalate, the number of positive turbid tubes was reduced presumably due to cell toxicity.

DISCUSSION

The results presented here demonstrate the practical value of microbial assay systems for the detection of genetically active chemicals in the tissue of the edible mussel, Mytilus edulis. We have found from selected sites around the coast of the United Kingdom that mantle tissues of mussels may contain chemicals which are extractable in ethanol and are capable of inducing mutation in bacteria and mitotic gene conversion in yeast. The presence of nutrients, such as free amino acids in the tissues of many marine species, prevents the uncritical use of the standard microbial mutagenicity assay systems with extracts of all marine organisms. However, with appropriate technical modifications, we have been able to utilize the techniques with a diverse range of marine organisms such as plankton, oysters, crabs, and a number of fish species.

The genetic techniques described here demonstrate only the presence of genetically active materials. Although we were able to show that at least one sample contains a predominantly frameshift mutagen, these techniques did not provide direct information of the chemical nature of the contaminating agent.

TABLE 10. GENETIC EFFECTS OF DI-ISO-OCTYL PHTHALATE AS MEASURED IN A SERIES OF FLUCTUATION TESTS USING CULTURES OF *ESCHERICHIA COLI* AND *SALMONELLA TYPHIMURIUM*

Strain	Concentration of phthalate in µgm/mℓ	No.of tubes sampled	No.of tubes positive		Significance
			Control	Treated	
Salmonella	1	50	13	18	NS
typhimurium	5	50	13	26	< .02
TA98	10	50	13	18	NS
TA98	1	96	2	9	< .05
	5	96	2	12	< .01
	10	96	2	6	NS
TA1538	1	96	2	9	< .05
	5	96	2	6	NS
	10	96	2	6	NS
TA100	1	96	19	18	NS
	5	96	19	27	NS
	10	96	19	17	NS
Escherichia coli	1	96	6	10	NS
343/113	5	96	6	23	< .001
lys 60	10	96	6	8	NS
343/113	1	96	14	19	NS
lys 60	5	96	14	18	NS
	10	96	14	10	NS

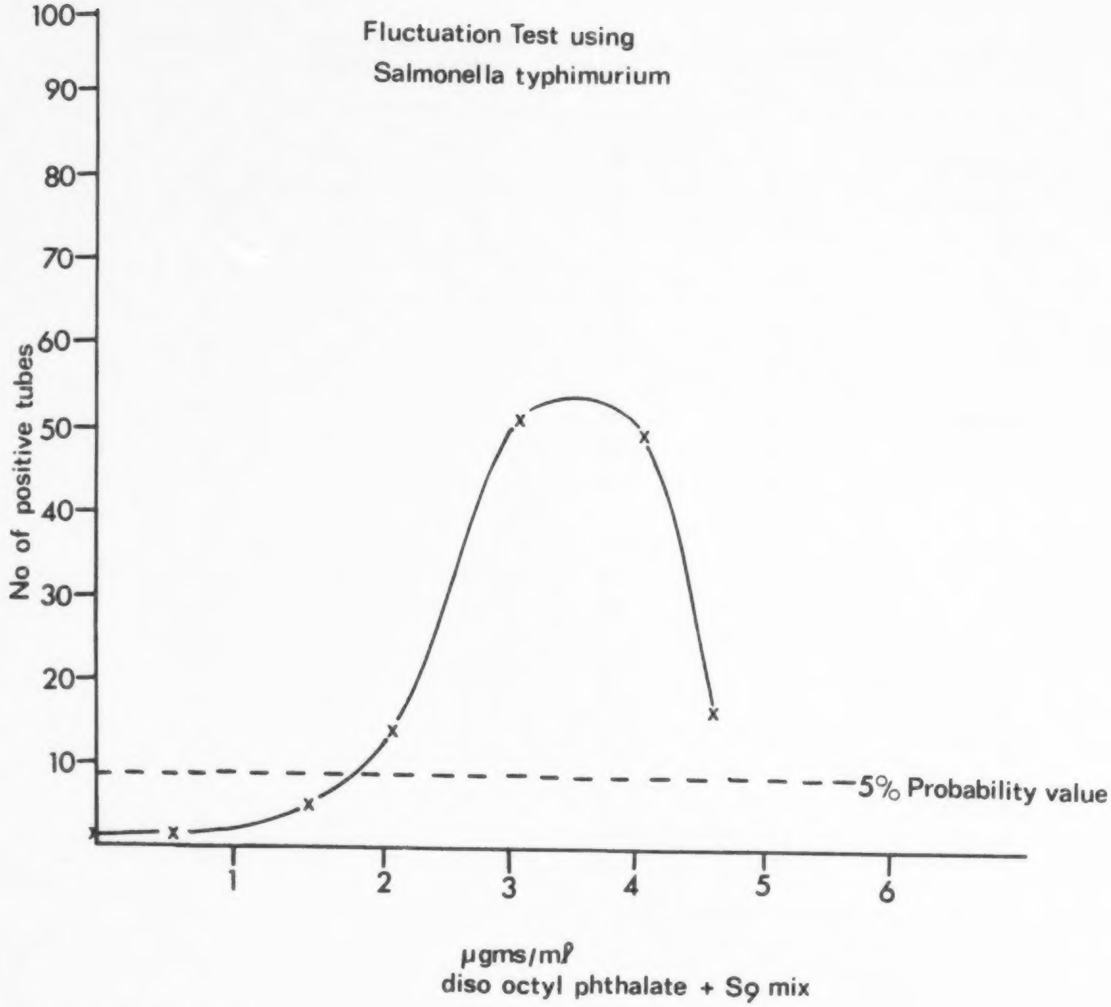

Figure 2. The effects of di-iso-octyl phthalate in the presence of S9 mix and co-factors upon the induction of turbid tubes produced by mutation to prototrophy in Salmonella typhimurium strain TA98. The test was performed with 96 well microtitre plates. The figure shows the number of positive tubes produced by concentrations of up to 5 μg/mℓ di-iso-octyl phthalate dissolved in 95% ethanol. All results above the dotted line are significant at the 5% level.

The individual mussel samples from the different sites probably contain a diverse collection of chemicals; it is unlikely that the activity we detected derives either from a single or group of chemicals. It is likely that each of the sites represent a different pool of genetically active chemicals that will require characterization. It is also unlikely that extraction of samples in only a single solvent (in our case, ethanol) would provide a complete extraction of all chemicals capable of inducing genetic change. However, extraction of mussel tissue with a variety of solvents demonstrated that the major proportion of detectable genetic activity was extractable with chloroform. Chemical analysis of the chloroform extract indiciated the presence of phthalate esters at concentrations as high as 11 ug per mussel.

Assays of the mutagenic potential of the one of the identifiable phthalate esters, i.e., di-2-ethylhexyl phthalate, demonstrated that the chemical was active in bacterial fluctuation tests both in the presence and absence of a rat liver microsome fraction. Experiments are still in progress to determine the mutagenic potential of the remaining chemicals detectable in the mussel extracts.

Our work demonstrated the presence in mussels of chemicals which cause mutation in microbes, but did not provide information on the potential of these chemicals to produce similar changes in the cells of higher organisms. However, our techniques do provide useful information on the chemicals in the marine environment which have the potential to produce genetic damage in organisms through direct exposure or via marine food chains. After the identification of the active chemicals by techniques we have described, the evaluation of their potential hazards will require the use of a range of test systems of more direct relevance to the organisms of concern. In this context, it is of interest that the extracts of mussels collected in the Mumbles area show predominantly frameshift mutagenic activity. In the case of such mutagens, studies of enzymatic variants of indicator species would produce only limited information on the amount of genetic change taking place because variants would be produced by predominantly base-change mutagens.

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BIPHENYL HYDROXYLASE ACTIVITY AND THE DETECTION OF CARCINOGENS

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ABSTRACT

The *in vitro* stimulation of biphenyl 2-hydroxylase activity by chemical carcinogens was examined for selected plant and animal microsomes by spectrophotofluorometric methods. An apparent increase in 2-hydroxybiphenyl was observed after preincubation of microsomes with carcinogens. In each case, the increase could be attributed to metabolites of the carcinogen which fluoresced at the same wavelength used to measure 2-hydroxybiphenyl. Quantification of biphenyl metabolite production using high pressure liquid chromatography to separate the compounds showed that preincubation of microsomes with chemical carcinogens had no effect on 2-hydroxybiphenyl formation and caused a decrease in 4-hydroxybiphenyl production. By using terphenyl as a substrate, a minimum of three different metabolites were formed *in vitro* by hamster microsomes as determined by high pressure liquid chromatography. One of these metabolites was not formed after preincubation of the microsomes with benzo(a)pyrene.

INTRODUCTION

Biphenyl and polychlorinated biphenyls are present in marine, freshwater, and terrestrial environments. The microsomal mixed function oxidases of a number of marine (Willis and Addison, 1974), freshwater (Creaven *et al*., 1965; Willis and Addison, 1974), and terrestrial (Creaven *et al*., 1965; Basu *et al*., 1971) animals detoxify biphenyl by formation of 4-hydroxybiphenyl as the major metabolite and 2-hydroxybiphenyl as a minor metabolite. Depending on the species examined, the ratio of 4-hydroxybiphenyl to 2-hydroxybiphenyl ranges from 26:1 to 2:1. Microbial metabolism of biphenyl has also been examined, and it has been shown that bacteria form dihydroxybiphenyls (Catelini *et al.*, 1970, 1971, 1973, Ioannides and Parke, 1975; Cerniglia *et al.*, 1978), whereas fungi form monohydroxybiphenyls (McPherson *et al.*, 1976; Dodge *et al.*,1978).

Pretreatment of animals with chemical carcinogens such as safrole, benzo(a)pyrene, or methylcholanthrene results in a selective stimulation of biphenyl 2-hydroxylase. Metabolite production is measured *in vitro* with purified microsomes from treated and untreated animals, biphenyl as a substrate, and an NADPH-regenerating system. The quantity of 2-hydroxybiphenyl and 4-hydroxybiphenyl formed, in most cases, is determined spectrophotofluorometrically (Creaven *et al.*, 1965). The amount of 2-hydroxybiphenyl formed by microsomes from treated animals is increased 2- to 20-fold over the untreated controls (Friedman *et al.*, 1972; Burke and Bridges, 1975; Nebert *et al.*, 1975; Atlas and Nebert, 1976; Burke and Prough, 1976; Tredger and Chhabra, 1976.) Pretreatment with phenobarbitone stimulates biphenyl 4-hydroxylase with no effect on biphenyl 2-hydroxylase (Burke and Bridges, 1975; Ioannides and Parke, 1975). The stimulation of biphenyl 2-hydroxylase by chemical carcinogens occurs in two phases: an initial activation of the enzyme followed by enzyme induction (McPherson *et al.*, 1976; Parke, 1976). It has been suggested that biphenyl 2-hydroxylase is associated with cytochrome P 448, whereas biphenyl 4-hydroxylase is associated primarily with cytochrome (Burke and Bridges, 1975; Burke and Prough, 1976; Parke, 1976).

Preincubation of animal and plant microsomes with chemical carcinogens has also been reported to selectively increase 2-hydroxybiphenyl formation. This *in vitro* stimulation results in a 60 to 300% increase in the amount of 2-hydroxybiphenyl (Burke and Bridges, 1975; McPherson *et al.*, 1974a, 1974b, 1975a, 1975b, 1975c, 1976). Non-carcinogens have no effect (McPherson *et al.*, 1974a), and 4-hydroxybiphenyl production is not affected. Storage destroys the ability of the microsomes to respond to the carcinogens (McPherson *et al.*, 1975a). Initial experiments employed (^{14}C) biphenyl and used radioisotopic methods to measure metabolite production (Burke and Bridges, 1975; McPherson *et al.*, 1975a). In subsequent work, the spectrophotofluorometric assay (Creaven *et al.*, 1965) was used exclusively.

The main objective of our work was to examine the feasibility of using the *in vitro* stimulation of biphenyl 2-hydroxylase by chemical carcinogens as a rapid prescreen for mutagenic and carcinogenic compounds to complement the presently available biological testing methods. Initial experiments were designed to duplicate exactly the work of others. We therefore examined the effect of known chemical carcinogens and non-carcinogens on *in vitro* biphenyl 2-hydroxylase, using microsomes from the mouse, rat, hamster, and avocado, *Persea americana*. Microsomes from the cauliflower and apple were also tested. In addition, we began a preliminary study of the *in vitro* metabolism of *m*-terphenyl by purified hamster microsomes.

MATERIALS AND METHODS

Chemicals

Benzo(a)pyrene (BaP) and safrole (SA) were purchased from Aldrich Chemical Co., α-naphthylamine (αNA) and β-naphthylamine (βNA) were from Sigma, and 20-methylcholanthrene (MC) was from K K Chemicals. All were of the highest purity available. These compounds were dissolved in peanut oil (Planters) to provide stock solutions at 1*mM*. Biphenyl (99.9 %pure, Ultrex) was obtained from J.T. Baker. It was dissolved in 1.5% (w/v) Tween 80 and 1.15% (w/v) KCl to make a 13 *mM* stock solution. The hydroxylated standards, 2-hydroxybiphenyl (99+%) and 4-hydroxybiphenyl (97%) were obtained from Aldrich Chemical Co. Stock solutions were made in 5% (v/v) aqueous ethanol at the following concentrations: 4-hydroxybiphenyl, 0.146 μ moles/mℓ; 2-hydroxybiphenyl, 0.0342 μ moles/mℓ; 2-hydroxybiphenyl and 4-hydroxybiphenyl mixture at 0.0342 and 0.146 μ moles/mℓ, respectively. A thin-layer chromatogram of the substrate and standard compounds at 0.1 mg material per spot showed no impurities in the biphenyl or 2-hydroxybiphenyl, but trace amounts of biphenyl and 2-hydroxybiphenyl were found in the 4-hydroxybiphenyl standard. Meta-terphenyl was recrystallized and a 13 *mM* stock solution in Tween 80 and 1.15% KCl made as for biphenyl. The *n*-heptane was "distilled in glass" from Burdick and Jackson Laboratories, and the succinic acid (99.4%) was obtained from J. T. Baker. All other chemicals were reagent grade.

Animals

Swiss-Webster mice (mean weight 37.9 g), Sprague-Dawley rats (mean weight 172.5 g), and Syrian hamsters (mean weight 118.7 g) were obtained from commerical breeders. Water and food (Wayne Lab Blocks) were provided *ad libitum*. Animals were sacrificed between 0830 and 1030 hr by decapitation.

Preparation of Hepatic Microsomes

Microsomes were prepared by the method of McPherson *et al*. (1976). Livers were rapidly removed into cold buffered KCl (1.15% w/v KCl, 0.3 *M* NaH_2PO_4, pH 7.6), blotted, weighed, and placed in fresh cold buffered KCl. The pooled, weighed livers were homogenized with a motor-driven teflon pestle using 10 strokes of 10 seconds each at 1200 rpm. The homogenate was diluted with cold buffered KCl to 250 mg tissue per mℓ of homogenate and centrifuged (2° C) for 10 min at 15,000 g in an IEC B20 centrifuge. In one case, this low speed pellet was resuspended in buffered KCl at 25 mg protein/mℓ and used in a hydroxylation experiment. The low speed supernatant was decanted and centrifuged (2° C) for 60 min at 104,000 g in a Beckman L2-65B ultracentrifuge. The supernatant was discarded and the pellet washed with cold buffered KCl, resuspended in cold buffered KCl, and again centrifuged (2° C) for 60 min at 104,000 g. The final pellets were resuspended in cold buffered KCl at a protein concentration of 10 mg/mℓ. Protein was determined by the method of Lowry *et al*. (1951).

Preparation of Plant Microsomes

Plant microsomes were prepared according to the method of McPherson et al. (1975b). Plant material was obtained 24 hr prior to use and stored in the cold. Cauliflower heads were soaked in cold water for 1 hr before storage to rehydrate the tissue. The mesocarp portion of both avocado and apple was used; rosettes of cauliflower were shaved from the head. The tissue was weighed, placed in cold phosphate buffer (0.1 M NaH_2PO_4 pH 7.4), and homogenized in either a Virtis homogenizer or a Waring blender. Tissues were homogenized at 0.5 to 2 g tissue per mℓ of phosphate buffer. The homogenate was filtered through muslin and centrifuged (2° C) for 20 min at 13,500 g in an IEC B20 centrifuge. The supernatant was decanted and centrifuged (2° C) for 90 min at 80,000 g in a Beckman L2-65B ultracentri- fuge. The pellets were resuspended in cold phosphate buffer and adjusted to 1 to 10 mg/mℓ protein with cold buffered KCl. Protein was determined by the method of Lowry et al. (1951).

Hydroxylation Reactions

Hydroxylation reactions were performed according to the method of McPherson et al. (1975b, 1975c, 1976). All reactions were carried out at 37° C in a shaking water bath at 100 cpm. The microsomal mixtures were warmed for 60 sec after addition of the NADPH-regenerating system. The 10-fold concentrated NADPH-regenerating system consisted of: glucose-6-phosphate dehydrogenase, 20 IU/mℓ; glucose-6-phosphate, 25 mM; NADP, 5 mM; $MgSO_4$, 0.5 mM dissolved in buffered KCl.

In the case of the crude homogenates, low speed supernatants and pellets, and plant microsomes, 1.8 mℓ of the preparation was used directly in the reaction mixture. The final protein concentration in the 2 mℓ reaction mixture for these preparations is given in Table 1. An aliquot (0.4 mℓ) of the first high speed pellets and purified animal microsomes (second high speed pellets) at 10 mg protein/mℓ was added to 1.4 mℓ of cold buffered KCl to provide a final protein concentration of 2 mg/mℓ in the 2 mℓ reaction mixture. Each tube received 0.2 mℓ of the 10-fold concentrated NADPH-regenerating system. Test compound (0.5 mℓ) in oil, or oil alone was added, and incubated for 10 min. Biphenyl (0.3 mℓ of 13 mM stock solution) was added, and incubation continued for an additional 5 min. The reaction was terminated by the addition of 1 mℓ of 4 M HCl to each tube. The incubation mixtures used in each hydroxylation experiment are given in Table 2. In one experiment using purified hamster microsomes, 0.3 mℓ pf 13 mM m-terphenyl was added as substrate in place of biphenyl.

Metabolite Extraction

The entire incubation and heptane extraction procedure was carried out in 20 mℓ glass tubes with teflon lined screw caps. Following addition of HCl and standards where indicated, the tubes were immediately extracted with 10 mℓ of n-heptane by mechanically shaking for 5 min (Creaven, et al., 1965). The tubes were centrifuged at 2000 rpm for 15 min and stored overnight in the cold. Tubes were allowed to return to ambient temperature,

TABLE 1. PROTEIN CONCENTRATIONS IN HYDROXYLATION EXPERIMENTS

Preparation	Protein Concentration* (mg/mℓ)
Mouse homogenate	29.7
Rat homogenate	27.0
Hamster homogenate	29.7, 35.9
Mouse low-speed pellet	22.5
Hamster low-speed supernatant	13.3
Avocado microsomes	5.8
Apple microsomes	1.0
Cauliflower microsomes	5.0, 9.1
Cauliflower low-speed supernatant	2.2

*Two numbers indicate the concentrations in two separate experiments. Protein is given as the final concentration in 2 mℓ of reaction mixture.

TABLE 2. INCUBATION MIXTURES USED IN HYDROXYLATION REACTIONS*

Microsomal** system	+ Oil		+ HCl***	
"	+ Oil	+ Biphenyl	"	
"	+ Test compound in oil		"	
"	+ Test compound in oil	+ Biphenyl	"	
"		+ Biphenyl	"	+ Test compound in oil
"	+ Oil		"	+ 2- or 4-Hydroxy-biphenyl + biphenyl

* Materials are listed in order of addition from left to right. The microsomal system and HCl were added to all tubes. A blank space indicates no addition, but continued incubation.

** The microsomal syustem consists of the microsomes or homogenate fractions plus the NADPH-regenerating system.

*** The HCl was added at the end of the incubation period to terminate the reaction.

and 2 mℓ of the heptane layer in each tube was shaken for 5 min with 10 mℓ of 0.1 *N* NaOH. The mixture was centrifuged for 10 min at 2000 rpm, 2 mℓ of the NaOH layer transferred to a cuvette, and adjusted to pH 5.5 by addition of 0.5 mℓ of 0.5 *N* succinic acid (Creaven *et al.*, 1965).

Analytical Methods

Fluorescence of the 2- and 4-hydroxbiphenyl compounds was determined by the method of Creaven *et al.*, (1965) using an Aminco-Bowman spectro photofluorometer with a high-pressure xenon lamp. The fluorescence-intensity of each sample was measured first for 4-hydroxybiphenyl by exciting at 270 to 272 nm and measuring emission at 335 nm, and then for 2-hydroxybiphenyl by exciting at 290 nm and measuring emission at 412 nm. Standard curves were constructed for each biological preparation for every experiment with dilutions of the tubes containing known concentrations of 2- and 4-hydroxybiphenyl. The fluorescence at wavelengths used to measure 2-hydroxybiphenyl was corrected for the contribution of 4-hydroxybiphenyl according to the method of Creaven *et al.*, (1965).

Metabolite separation was accomplished by a Perkin-Elmer 220 high pressure liquid chromatograph (HPLC) with a Partisil$^{\text{TM}}$ PAC (Reeve Angel) column of 25 cm x 46 mm inside diameter, The solvent was 85% *n*-hexane, 15% tetrahydrofuran (no preservatives) at a flow rate of 2 mℓ per min. Column pressure was 300 psi, and the temperature was ambient. The chromatograph was attached to the spectrophotofluorometer by means of 150 μℓ flowthrough cell having a 2 mm path length. Fluorescence was measured for biphenyl metabolites by exciting at 300 nm and recording emission a 335 nm. Terphenyl metabolites were examined at several different excitation-emission wavelengths. The location of compounds in the chromatographic fractions was recorded by a Linear Instrument Co. strip chart recorder at a chart speed of 16 inches per hr. All chromatograms were recorded at range 0.33.

The *n*-heptane layer (10 μℓ) from each sample was injected directly into the HPLC. Quantitation of peak heights was accomplished by constructing standard curves with three different concentrations of 2-hydroxybiphenyl and 4-hydroxybiphenyl ranging from 1 to 5 ng per injection. Calibration curves were based on peak height because repeated injections showed sufficient reproducibility (in retention time and peak width) to alleviate the need for area measurements. The limit of detection was determined to be approximately 1 ng for 4-hydroxybiphenyl and 0.5 ng for 2-hydroxybiphenyl.

RESULTS

Measurement of Biphenyl Metabolites by Fluorometric Analysis

The results (Tables 3 and 4) were obtained from several experiments designed to either duplicate previously reported work (Burke and Bridges, 1975; McPherson *et al.*, 1974a, 1974b, 1975a, 1975b, 1975c, 1976) or to examine microsomes from other sources with respect to activation of biphenyl 2-hydroxylase. The quantity of 2-hydroxybiphenyl and 4-hydroxybiphenyl in each of the reaction mixtures was determined fluorometrically by the method of Creaven et al. (1965). The known carcinogen, BaP, was included in all experiments as a positive control, and where possible, αNA was included as an example of a non-carcinogen. All other test compounds are known carcinogens (McCann et al., 1975) and were used when the supply of microsomes was sufficient.

It was apparent that the oil used as the solvent for test compounds contributed fluorescence at the wavelengths used to measure both hydroxylated biphenyls. However, the oil was present in all reaction tubes, including those containing the 2- and 4-hydroxybiphenyl standards. Fluorescence contribution of the oil was incorporated into the standard curves used to obtain quantitative data; therefore subtraction from values obtained for the experimental incubation mixtures was not required.

Table 3 shows the results of three different hydroxylation experiments using various fractions of hepatic homogenates from mice, rats, and hamsters. Comparison of the amount of 2-hydroxybiphenyl produced in the oil plus biphenyl reaction mixture with that produced in the carcinogen plus biphenyl reaction mixture indicated that there was an apparent stimulation of 2-hydroxybiphenyl production of 123 to 3500% in the presence of carcinogen. However, extracts of incubation mixtures containing test compound alone showed a significant fluorescence at the wavelength used to determine 2-hydroxybiphenyl, and a smaller fluorescence at the wavelength used to determine 4-hydroxybiphenyl. In nearly all cases, the apparent increase in the amount of 2-hydroxybiphenyl produced in the presence of carcinogen could be eliminated by subtracting the fluorescence contributed by the metabolites of the test compound.

The effect of various test compounds on hydroxybiphenyl production by plant microsomes was also examined because McPherson et al. (1975b, 1975c) had reported that 3,4-benzopyrene stimulates 2-hydroxybiphenyl formation by avocado microsomes. The results (Table 4) using plant microsomes are similar to those obtained with animal microsomes. In this case, metabolites of the test compound contributed significantly to the determination of both hydroxybiphenyl compounds.

The fluorescence excitation and emission spectra of extracts from some of the incubation mixtures were examined by fluorometric assay (Creaven et al., 1965). The spectra for the two hydroxylated standards are shown in Figure 1. The 2-hydroxybiphenyl standard showed an emission peak at 412 nm, and 4-hydroxybiphenyl had a major emission peak at 335 nm with a minor peak in the vicinity of 412 nm in agreement with the data of Creaven et al., (1965).

Material extracted from incubation mixtures containing purified hamster microsomes and oil plus biphenyl showed (Figure 2A) a shift in the emission peak for 2-hydroxybiphenyl to 418 nm and a broad shoulder in the region used to measure 4-hydroxybiphenyl (335 nm). Incubation mixtures to

TABLE 3. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4-HYDROXBIPHENYL AND 2-HYDROXYBIPHENYL BY LIVER FRACTIONS

			n mole min^{-1} mg protein^{-1}		
Fraction	Animal	Reaction mixture	4-Hydroxybiphenyl	2-Hydroxybiphenyl	Corrected*
Crude homogenate	Mouse	Oil	0.007	1.05	
		BaP	0.014	1.41	
		Oil + biphenyl	0.026	2.55	
		Biphenyl + BaP**	0.033	1.03	
		BaP + biphenyl	0.005 (19%)***	1.58 (62%)	0.17
	Rat	Oil	0.016	0.19	
		BaP	0	0.54	
		Oil + biphenyl	0.018	0.34	
		Biphenyl + BaP	0.024	0.86	
		BaP + biphenyl	0.011 (61%)	0.92 (271%)	0.38
	Hamster[†]	Oil	0.004	0.19	
		BaP	0	0.37	
		SA	0.006	0	
		Oil + biphenyl	0.022	0.29	
		Biphenyl + BaP	0.018	0.22	
		Biphenyl + SA	0.034	0.005	
		BaP + biphenyl	0.007 (32%)	0.78 (269%)	0.41
		SA + biphenyl	0.021 (96%)	0 (--)	0
Low-speed supernatant	Hamster	Oil	0.016	0.008	
		BaP	0.011	0.020	
		Oil + biphenyl	0	0.020	
		Biphenyl + BaP	0.142	0.122	
		BaP + biphenyl	0.055 (--)	0.075 (375%)	0.055
Low-speed pellet	Mouse	Oil	0.005	0.003	
		BaP	0.009	0.008	
		Oil + biphenyl	0.032	0.005	
		Biphenyl + BaP	0.045	0.006	
		BaP + biphenyl	0.032 (100%)	0.008 (160%)	0
First high-speed pellet	Hamster	Oil	--[‡]	--	
		BaP	0	0.56	
		SA	0	0.07	
		MC	0	0.37	
		Oil + biphenyl	0.47	0.26	
		Biphenyl + BaP	--	--	
		Biphenyl + SA	1.58	0.85	
		Biphenyl + MC	1.32	1.43	
		BaP + biphenyl	0.51 (108%)	0.54 (208%)	0
		SA + biphenyl	0.66 (140%)	0.32 (123%)	0.25
		MC + biphenyl	0.56 (119%)	0.65 (250%)	0.28
Second high-speed pellet	Mouse[§]	Oil	0.21	0.06	
		BaP	0.09	0.10	
		SA	0.82	0	
		MC	0.29	0.12	
		αNA	0.26	0.07	
		βNA	0.07	0.29	
		Oil + biphenyl	2.54	0.008	
		Biphenyl + BaP	2.23	0.13	
		Biphenyl + SA	3.85	0	
		Biphenyl + MC	2.68	0.26	
		Biphenyl + αNA	3.12	0.03	
		Biphenyl + βNA	3.38	0.04	
		BaP + biphenyl	2.86 (113%)	0.034(425%)	0
		SA + biphenyl	1.15 (45%)	0.07 (875%)	0.07
		MC + biphenyl	4.00 (158%)	0.03 (375%)	0
		αNA + biphenyl	2.97 (117%)	0 (--)	0
		βNA + biphenyl	1.10 (43%)	0.28 (3500%)	0

TABLE 3. (CONTINUED)

Second high speed pellet	Rat	Oil	0.54	0	
		BaP	0.13	0.10	
		Oil + biphenyl	1.25	0.015	
		Biphenyl + BaP	1.10	0.044	
		BaP + biphenyl	1.01 (81%)	0.074 (493%)	0
	Hamster[§]	Oil	0.12	0.008	
		BaP	0.15	0.20	
		SA	0.62	0.06	
		MC	0.25	0.42	
		αNA	0.21	0.13	
		βNA	0.71	0.58	
		Oil + biphenyl	0.93	0.17	
		Biphenyl + BaP	1.46	0.40	
		Biphenyl + SA	2.08	0.23	
		Biphenyl + MC	1.29	0.44	
		Biphenyl + αNA	1.57	0.68	
		Biphenyl + βNA	1.72	0.65	
		BaP + biphenyl	0.78 (84%)	0.32 (188%)	0.12
		SA + biphenyl[#]	1.07 (115%)	0.18 (106%)	0.12
		MC + biphenyl[#]	1.03 (111%)	0.30 (176%)	0
		αNA + biphenyl[#]	1.04 (112%)	0.28 (165%)	0.15
		βNA + biphenyl[#]	0.78 (84%)	0.56 (329%)	0

[*] The contribution of test compound metabolites as determined in reaction mixtures containing test compound alone was subtracted from the quantity of 2-hydroxybiphenyl apparently present in the test compound plus biphenyl reaction mixtures.

[**] When the test compound follows the substrate, it was added to the reaction mixture after the addition of HCl.

[***] Numbers in parentheses are results expressed as percent relative to control values.

[†] Results are the average of two experiments for the oil, oil and biphenyl, and BaP mixtures.

[‡] A dashed line means that the results were not determined because the tube was lost.

[§] Results are tha average of two experiments, 2 to 3 replicates for the oil, oil and biphenyl, and BaP mixtures.

[#] Results are the average of two replicates in one experiment.

TABLE 4. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4-HYDROXYBIPHENYL AND 2-HYDROXYBIPHENYL BY PLANT MICROSOMES*

Fraction	Plant	Reaction mixture	n mole·min^{-1} mg protein^{-1}			
			4-Hydroxybiphenyl	Corrected	2-Hydroxybiphenyl	Corrected
Low speed supernatant	Cauliflower	Oil	0.53		0.27	
		BaP	0.80		0.39	
		SA	0.80		0.18	
		Oil + biphenyl	0.27		0.18	
		Biphenyl + BaP	0.49		0.66	
		BaP + biphenyl	0.43	0	0.31	0
		SA + biphenyl	0.61	0	0.12	0
Purified microsomes	Cauliflower**	Oil	0.18		0.085	
		BaP	0.21		0.075	
		SA	0.25		0.032	
		MC	0.036		0.039	
		Oil + biphenyl	0.15		0.003	
		Biphenyl + BaP	0.065		0.026	
		Biphenyl + SA	0.081		0	
		Biphenyl + MC	0.048		0.27	
		BaP + biphenyl	0.12	0	0.032	0
		SA + biphenyl	0.21	0	0.012	0
		MC + biphenyl	0.048	0.012	0.036	0
	Avocado	Oil	0.102		0.010	
		BaP	0.082		0.031	
		Oil + biphenyl	0.077		0.005	
		Biphenyl + BaP	0.113		0.031	
		BaP + biphenyl	0,087	0.005	0.005	0
	Apple	Oil	0.34		0	
		BaP	0.31		0.31	
		Oil + biphenyl	0.54		0	
		Biphenyl + BaP	0.57		0.20	
		BaP + biphenyl	0.43	0.12	0	0

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* Symbols and procedures are described in Table 3.

**All except MC are the average of results from two experiments.

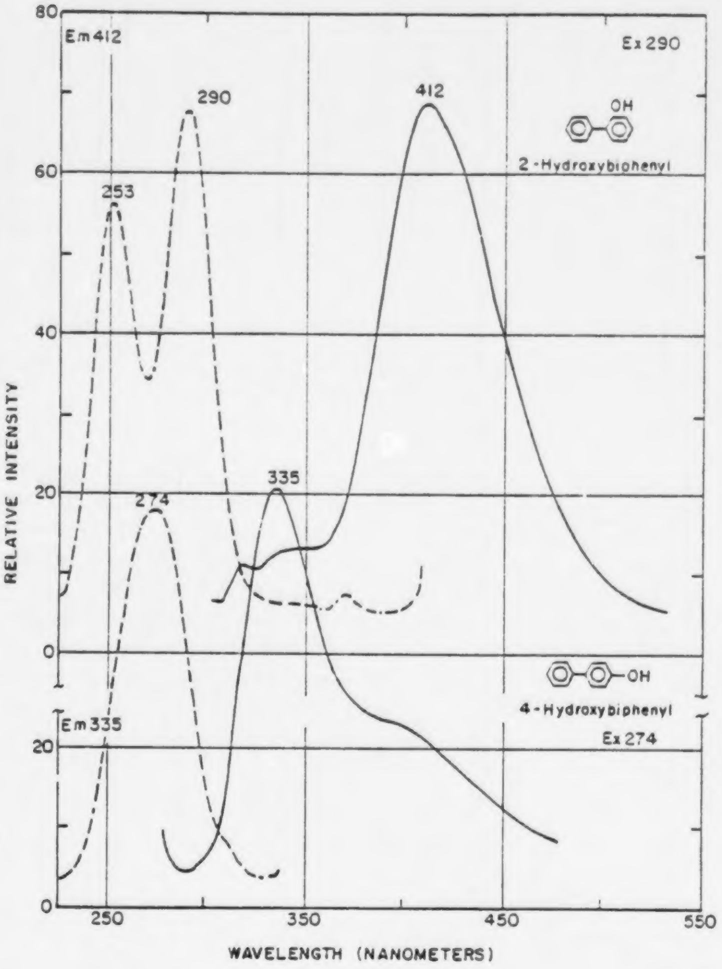

Figure 1. Excitation and emission spectra of 2- and 4-hydroxybiphenyl standards. Dashed lines are excitation spectra with emission measures at 412 nm and 335 nm for the 2- and 4-hydroxybiphenyl standards, respectively. Solid lines are emission spectra with excitation at 290 nm and for 2- and 4-hydroxybiphenyl, respectively. The 2-hydroxybiphenyl spectrum is vertically offset and is shown in the upper portion as in this and all subsequent figures.

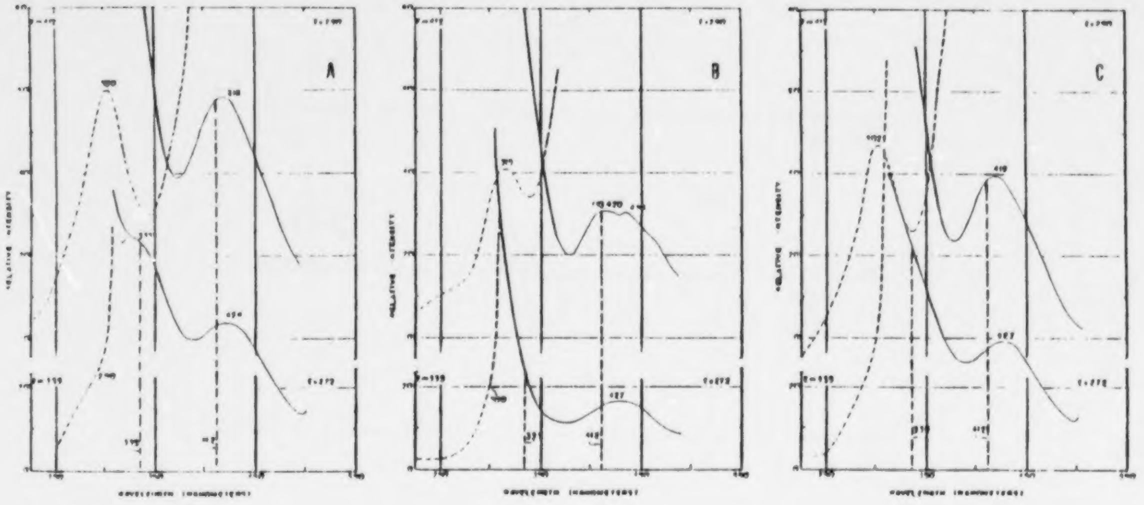

Figure 2. Excitation and emission spectra of material extracted from incubation mixtures containing purified hamster microsomes and (A) oil plus biphenyl; (B) BaP in oil; (C) Bap in oil plus biphenyl. See Figure 1 for a description of symbols. Dashed vertical lines designate 335 and 412 nm, the wavelengths at which emission is measured in the fluorometric assay.

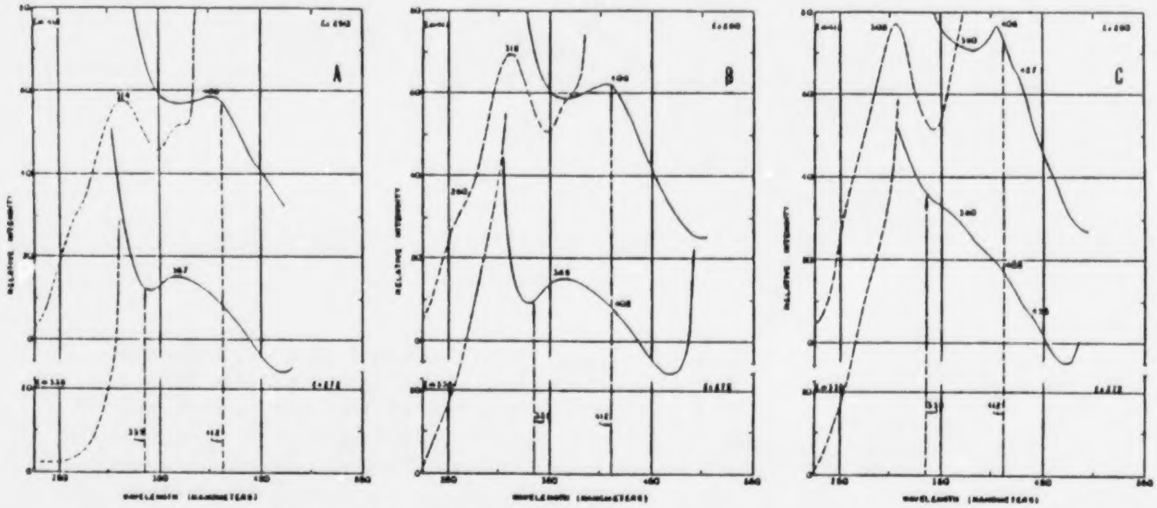

Figure 3. Excitation and emission spectra of material extracted from incubation mixtures containing purified cauliflower microsomes and (A) oil plus biphenyl; (B) BaP in oil; (C) BaP in oil plus biphenyl. For a description of the symbols, see Figures 1 and 2.

which BaP in oil had been added (Figure 2B) contained material which had a broad emission peak from approximately 380 to 470 nm after excitation at 290 nm. The emission at 335 nm (excitation at 272 nm) was part of the excitation scatter peak. In mixtures containing BaP in oil plus biphenyl (Figure 2C), material produced also had the 418 nm peak, but lacked the 335 nm shoulder.

Plant microsomal (Figures 3, 4) material extracted from the incubation mixtures containing oil plus biphenyl showed two emission optima (Figure 3A). Excitation at 290 nm gave an emission peak at 405 nm, and at 272 nm, an emission peak at 367nm. A similar pattern was oberved in material from microsomes incubated with BaP in oil (Figure 3B) or MC in oil (Figure 4A). Incubation mixtures containing both carcinogen and biphenyl yielded material which had an increased amount of fluorescence in the 405 to 410 nm region. The emission spectrum obtained by exciting at 272 nm showed essentially no discrete peaks in the case of BaP, and a broad 350 to 450 nm shoulder in the case of MC.

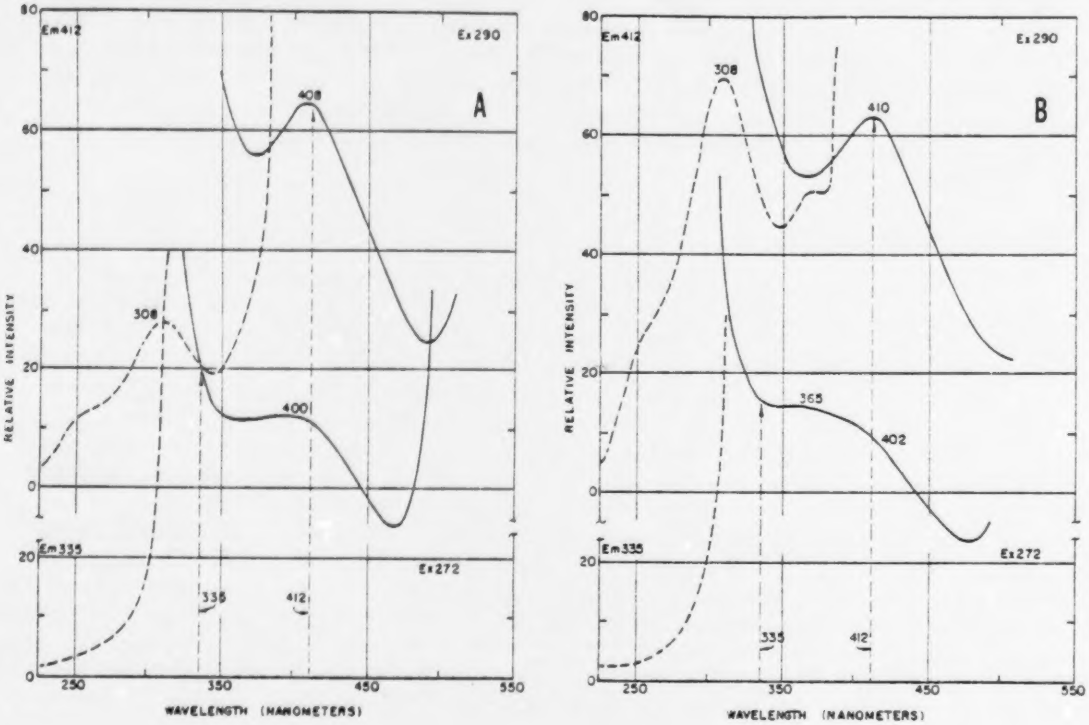

Figure 4. Excitation and emission spectra of material extracted from incubation mixtures containing purified cauliflower microsomes and (A) MC in oil; (B) MC in oil plus biphenyl.

Analysis of Biphenyl Metabolites using HPLC

An alternative method which involved separation of metabolites by high pressure liquid chromatography and detection by fluorometry was used to determine the amount of 2- and 4-hydroxybiphenyl produced in incubation mixtures containing hamster microsomes. Figure 5 shows that this method allows complete separation of the two hydroxylated biphenyls in a mixture of pure compounds. The average retention time was 9.0 min for 2-hydroxybiphenyl and 11.7 min for 4-hydroxybiphenyl.

Figure 6 shows chromatograms of material obtained from incubation mixtures which contained purified hamster microsomes. Microsomes incubated with oil plus biphenyl produced easily detectable amounts of 2- and 4-hydroxybiphenyl (Figure 6A). The material eluting immediately after injection was derived from the oil (Figure 6B) and was present in all samples. Figure 6C shows a typical chromatogram of a sample from an incubation mixture containing BaP in oil plus biphenyl.

Table 5 presents quantitative data obtained from these chromatograms. It can be seen that the test compounds did not contribute fluorescent material to the 2- and 4-hydroxybiphenyl peaks under the conditions of this assay. The amount of 2- and 4-hydroxybiphenyl produced in the reaction containing substrate alone agrees with the quantity determined using the fluorometric method (Table 3). However, in the presence of test compound, the amount of 4-hydroxybiphenyl apparently decreased in all but one case, and the amount of 2-hydroxybiphenyl remained constant or decreased slightly.

Terphenyl Metabolism

Purified hamster microsomes were used in an experiment designed to examine the metabolites of terphenyl produced *in vitro*. The effect of BaP on *m*-terphenyl metabolite production was also investigated. Incubation conditions were identical to those used for biphenyl. The *n*-heptane extract from these mixtures was chromatographed by HPLC as described for biphenyl. A number of different excitation and emission wavelengths were used (Table 6) to detect possible metabolites of terphenyl. In each case (Figure 7), unaltered terphenyl was observed at an average retention time of 3.1 min. A total of three different metabolites were detected at two different excitation and emission wavelengths (Table 6). A chromatogram showing the first two metabolites is presented in Figure 7A. A reaction mixture containing terphenyl as a substrate which had been preincubated for 10 min with BaP was also examined at an excitation wavelength of 270 nm and and an emission wavelength of 350 nm. The metabolite with a retention time of 14.2 to 14.4 min was absent, and the metabolite with a retention time of 16.2 to 16.3 min had a reduced peak height (Figure 7B).

TABLE 5. EFFECT OF TEST COMPOUNDS ON PRODUCTION OF 4- and 2-HYDROXYBIPHENYL AS DETERMINED BY QUANTITATIVE HPLC

Reaction mixture*	n mole min^{-1} mg $\mathrm{protein}^{-1}$ 4-Hydroxybiphenyl	 2-Hydroxybiphenyl
First high speed pellet		
Oil + biphenyl	0.64	0.16
BaP + biphenyl	0.36	0.14
SA + biphenyl	0.26	0.20
MC + biphenyl	0.55	0.21
BaP	0.00	0.00
Second high speed pellet		
Oil + biphenyl	0.59	0.22
BaP + biphenyl	0.26	0.15
SA + biphenyl	0.19	0.17
MC + biphenyl	0.24	0.16
αNA + biphenyl	0.30	0.18
βNA + biphenyl	0.14	0.09
Oil	0.00	0.00
BaP	0.04	0.02

* Hamster microsomes were used.

TABLE 6. METABOLITES OF TERPHENYL PRODUCED BY PURIFIED HAMSTER MICROSOMES

Wavelength (nm)	Peak heights (mm)* at R_t of: 14.2-14.4 min	 16.2-16.3 min	 17.8 min
Ex 270, Em 360	5.8	14.8	--**
Ex250, Em 360	2.2	5.5	--
Ex 270, Em 350	5.4	15.0	--
Ex 300, Em 360	--	7.0	9.0
Ex 300, Em 335	--	3.0	4.0

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* Peak widths as half-height were identical for each metabolite. Symbols used are: R_t = retention time, Ex = excitation, Em = emission.

**Not Present.

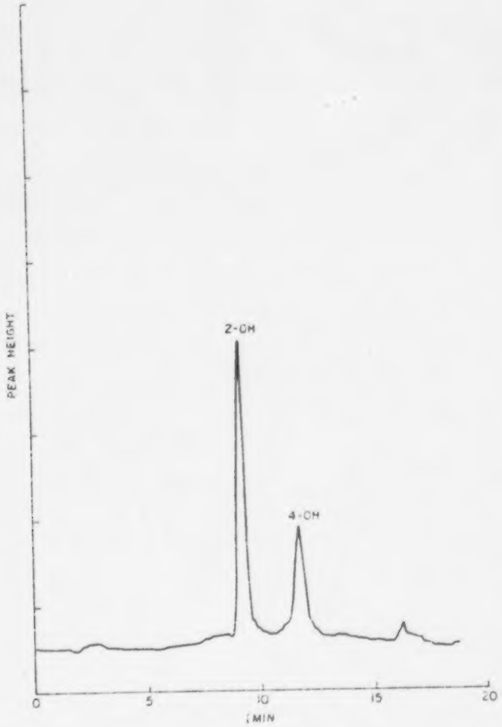

Figure 5. Separation of 2- and 4-hydroxybiphenyl standard by HPLC. The sample contained 2.4 ng of each pure compound. For details of the procedure, see Materials and Methods.

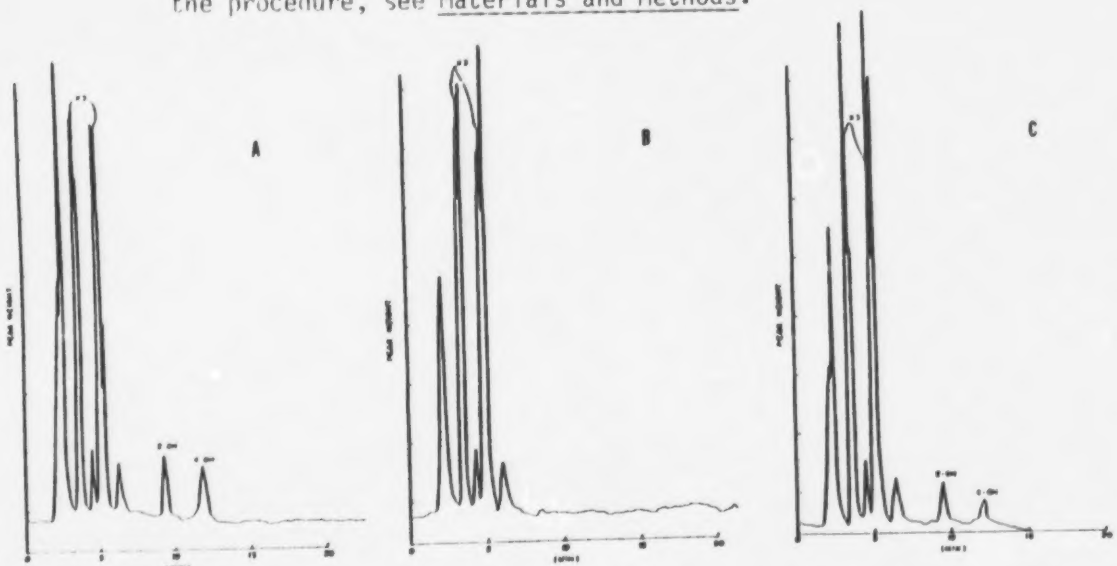

Figure 6. Material separated by HPLC and obtained from reaction mixtures containing purified hamster microsomes and (A) oil plus biphenyl; (B) oil alone; (C) BaP in oil plus biphenyl. For experimental details, see Materials and Methods.

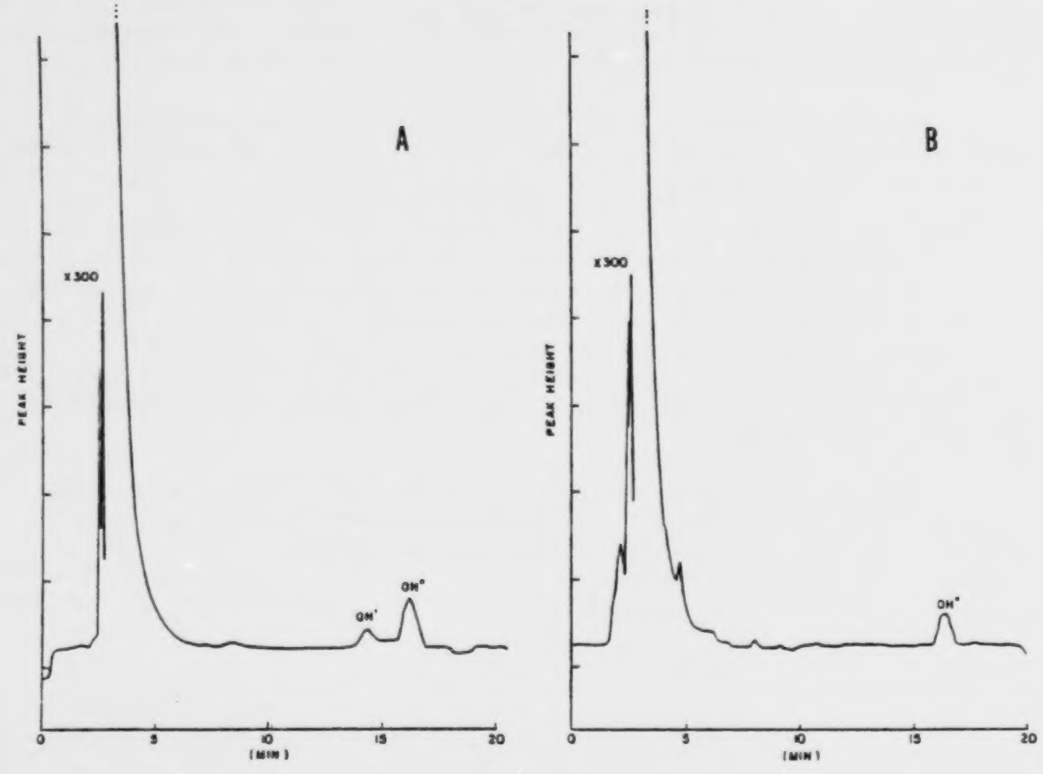

Figure 7. Material separated by HPLC obtained from reaction mixtures containing purified hamster microsomes and (A) oil plus *m*-terphenyl; (B) BaP in oil plus *m*-terphenyl. The excitation wavelength was 270 nm and the emission wavelength was 350 nm.

DISCUSSION

As reported by others (Burke et al., 1977; Tong et al., 1977a), the data presented in our paper demonstrate that the fluorometric determination of 2-hydroxybiphenyl production in vitro in the presence of carcinogens may result in spuriously high values. The data (Tables 3, 4; Figures 2,3, and 4) indicate that much, if not all, of the increase in fluorescence at 412 nm (the wavelength used to measure 2-hydroxybiphenyl) may be attributed to fluorescence of test compound metabolites. In addition, the emission peak for 2-hydroxybiphenyl changed from the expected 412 nm to 418 nm in the case of animal microsomes, and 405 nm in the case of plant microsomes. Fluorescence of the 4-hydroxybiphenyl compound at 335 nm became part of the excitation scattering peak in all microsomal extracts. Measurements made at 412 and 335 nm, therefore, are subject to considerable error.

Using a high pressure liquid chromatography system which permits unequivocal identification and quantification of the hydroxylated biphenyls, we were not able to demonstrate an in vitro stimulation of biphenyl 2-hydroxylase in contrast to the findings of others (Creaven et al., 1965; McPherson et al., 1974a, 1974b, 1975a, 1975b, 1975c, 1976; Tredger and Chhabra, 1976; Tong and Parke, 1977b). The data in Table 5 show that the amount of 4-hydroxybiphenyl decreased in the presence of test compounds. The amount of 2-hydroxybiphenyl remained constant or decreased (βNA). The discrepancy between these results and those of Burke and Bridges (1975) and McPherson et al. (1975a) who reported stimulation of biphenyl 2-hydroxylase by chemical carcinogens in vitro, using (^{14}C)biphenyl as a substrate is presently unexplained.

Terphenyl was metabolized in vitro to at least three products by purified hamster microsomes. The formation of one of these products is prevented by preincubation of the microsomes with BaP. Further work is necessary to determine whether or not this substrate may prove useful in the development of an in vitro microsomal screen for chemical carcinogens.

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PETROLEUM AND PETROLEUM COMBUSTION BYPRODUCTS AS POTENTIAL SOURCES OF MARINE ENVIRONMENTAL MUTAGENS

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ABSTRACT

We previously reported that polycyclic organic enriched fractions of used engine oil were mutagenic in the Ames *Salmonella* assay. Recent studies on a variety of oil samples obtained from automobile service stations in the St. John's area has demonstrated that used engine oils consistently contain mutagenic material, presumably polycyclic organic compounds. A mutagenic response has also been obtained with extracts of a sample of used, but not with extracts of a comparable brand of unused, non-petroleum base, synthetic engine oil. Circumstantial evidence is presented to suggest that the mutagens found in used engine oils originate from fuel combustion. In contrast to the positive results obtained with used engine oils, mutagenic activity has not been obtained with any of several different types of petroleum or unused engine oils. The possibility that polycyclic organic compounds originating from combustion processes could be a major anthropogenic source of marine environmental mutagens is briefly discussed.

INTRODUCTION

Environmental carcinogenesis is not only a human health concern, but has become an area of prime interest in all environmental studies, including aquatic toxicology. Polycyclic organic compounds (POC) are major air and water pollutants and are receiving considerable attention because a few have been shown to be both mutagenic and carcinogenic. An increase in the number of oil tanker spills has focused interest on the possible effects of elevated concentrations of POC on marine organisms. Major new energy technologies including coal combustion, liquefaction, and hydrogenation, as well as extensive tar sand extraction, will likely result in an increased contamination of the aquatic environment by polycyclic organic compounds.

Urban-associated wastewater and atmospheric fallout are probably the chief routes by which complex pollutant mixtures originating from industrial, domestic, and natural sources enter the aquatic environment. Used engine oil is a common pollutant in municipal wastewater (Farrington and Quinn, 1973; Tanacredi, 1977) and we have recently demonstrated that used oil contains mutagenic compounds (Payne *et al*., 1978). This report summarizes further studies on a variety of sources of potentially mutagenic POC which may enter waterways via oil spills, land runoff, or municipal wastewater.

Petroleum Hydrocarbon Mutagenicity

The method used to assess mutagenic activity in POC enriched fractions of petroleum hydrocarbons was previously reported (Payne *et al*., 1978). Oil was extracted with an equal volume of dimethyl sulfoxide (DMSO) and titers of this extract were used directly, with and without liver enzyme activation (9000 x g supernatant fraction of S9), in the Ames *Salmonella* assay (Ames *et al*., 1975). DMSO is a good solvent for extracting polycyclic organic compounds from aliphatic material (Natusch, 1978), and a number of compounds found in POC enriched fractions of a variety of petroleum oils are metabolized by fish liver S9 preparations (Table 1).

Crude petroleum likely contains hundreds or thousands of different polycyclic compounds and we have not yet begun to fractionate any oils into chemical "classes" for mutagenesis testing. There are limitations to testing complex mixtures of similar chemicals, such as the possibility for producing synergistic or antagonistic effects, but such effects may be of key biological importance. For instance, substrate competition has been suggested for the apparent inability of the potent carcinogen, benzo(a)pyrene, to form tumors when applied in combination with other hydrocarbon compounds (Schmeltz *et al*., 1978). Conversely, supraadditive effects can also be postulated when two or more chemicals are being tested in combination (Fingl and Woodburg, 1975).

TABLE 1. METABOLISM OF PETROLEUM BY FISH LIVER HOMOGENATES

Source	Band	Number of Metabolites Detected	RF. value
Kuwait crude	1	3	.30 .40 .50
	3	1	.40
	2	3	.30 .50 .60
Louisiana crude	3	3	.25 .50 .55
	2	3	.16 .40 .50
	1	2	.30 .50
No. 2 fuel oil	1	3	.17 .40 .50
	3	2	.40 .76
	2	2	.24 .50
Sable Island crude	3	2	.23 .50
	1	2	.30 .50
	2	4	.20 .30 .44 .50

Polycyclic aromatic compounds were extracted from petroleum with DMSO. These extracts were streaked on silica-gel plates which were developed in hexane:benzene (18:2). Fluorescent bands were eluted with methylene chloride which was evaporated under nitrogen, and the residues were dissolved in 0.1-0.5 mℓ of methanol. These residues were substituted for benzo(a)pyrene in our regular aryl hydrocarbon hydroxylase assay (Payne and Penrose, 1975). Hexane extracts were evaporated under nitrogen to approximately 100 μℓ and this volume was streaked on silica-gel plates which were developed in benzene:methanol (18:1) in the dark. Fluorescent metabolites were detected under UV.

Crude petroleum obtained from a variety of world sources, as well as some refined oils, including several brands of unused engine oil have now been assessed for mutagenic activity. Assays producing at least twice the background numbers of revertant colonies were considered to be positive. It is of special interest that mutagenicity has not been reliably demonstrated in POC extracts of any crude or refined oils assessed to date (Table 2). Table 3 presents some representative values obtained with four different types of petroleum including Pembina, Atkinson's Point, Norman Wells, and Alaskan crudes. In contrast to the negative results obtained with petroleum and refined oils, used engine oil collected from a number of automobile service stations in the St. John's area has consistently demonstrated a significant level of mutagenic activity. Most of the activity found in the DMSO extracts of used engine oil is dependent on enzyme activation, and this can be effected with either fish or rat liver tissue preparations. Enyzme/mediated mutagenicity has also been demonstrated with various Salmonella strains including TA 98, TA 100, TA 135, and TA 1538. Approximately one-fifth to one-quarter of the activity found in some samples of used oil does appear, however, to be due to the presence of direct acting mutagens.

To date, mutagenic activity has only been observed in extracts of used engine oil. This suggests that the mutagenic principal(s) originates from gasoline combustion or is generated from specific engine oil compounds within the crankcase. Mutagenic activity has also been detected in POC extracts of a sample of used, non-petroleum base, synthetic engine oil, but not in extracts of a comparable brand of unused oil (Table 4). Since petroleum-base and synthetic engine oils differ in composition, it is likely that the source of the mutagens found in both types is gasoline combustion. Further supportive evidence has come from analysis of samples of soot obtained from a car carburetor. This soot was observed to be mutagenic and POC-enriched fractions of soot, as well as both types of used engine oils, displayed similar fluorescent profiles on thin layer chromatograms. Also, most of the mutagenic activity associated with all three has been shown to be found in a band of material which migrates with a common Rf.

DISCUSSION

Used engine oils which may enter the sea via runoff and municipal wastewater could be an important source of marine environmental mutagens. It would appear that engine oil mutagenesis is a combustion-derived phenomenon and petroleum hydrocarbons (excellent sources of POC) which enter the ocean via accidental discharge could represent a minor source of mutagenic material (even if some proportions were mutagenic) compared with the potential offered by various combustion processes. For instance, approximately 8 million barrels of oil are lost yearly via accidental discharge into the world's oceans (calculated from a review by McAuliffe, 1976), whereas 6 million barrels of fuel are combusted daily by vehicular highway traffic in the United States (calculated from NAS, 1972). Also, vehicular oil consumption likely includes only a small proportion of the total combustion "budget" of any industrialized country.

It is of special interest that studies on hydrocarbon cores from the northwest Atlantic have demonstrated that urban air fallout is the most likely source of complex hydrocarbon mixtures found in coastal and continental sediments (Farrington *et al.*, 1977). All in all, the present evidence suggests that polycyclic aromatic material originating from combustion processes could be a major anthropogenic source of marine environmental mutagens.

TABLE 2. PETROLEUM HYDROCARBON MUTAGENICITY

Crude oils	Refined oils
Kuwait	Fuel Oil No. 2
Louisiana	Venezuelan Bunker
Western Canada	Texaco Motor Oil
Venezuelan	Esso Motor Oil
Norman Wells	Irving Motor Oil
Atkinson's Point	Gulf Motor Oil
Sable Island	Veedol Motor Oil
Alberta Tar Sands	
Alaskan	
Pembina	

Samples were extracted with DMSO in a 1:1 ratio and the polycyclic aromatic fraction obtained was used directly in the Ames assay with *Salmonella* strain TA 98. Extracts were tested in volumes ranging from 10 to 100 μℓ or more.

TABLE 3. PETROLEUM HYDROCARBON MUTAGENICITY

Petroleum	TA 98 Revertants
Pembina	
20 μℓ + S9	68, 55
100 μℓ + S9	40, 41
Atkinson's Point	
20 μℓ + S9	53, 57
100 μℓ + S9	51, 51
Norman Wells	
20 μℓ + S9	67, 62
100 μℓ + S9	41, 46
Alaskan	
20 μℓ + S9	84, 76
100 μℓ + S9	34, 37
Used Engine Oil (Positive Control)	
10 μℓ + S9	770, 729
Bacteria + S9 (Control)	47, 50

Petroleum was extracted with DMSO in a 1:1 ratio and the polycyclic aromatic fraction obtained was used directly in the Ames assay with Salmonella strain TA 98. Duplicate plate counts for a typical experiment are presented.

TABLE 4. MUTAGENICITY OF USED (SYNTHETIC) MOTOR OIL

Test Conditions	TA 98 Revertants
Bacteria + S9	49, 47
Unused Oil (100 μℓ)	27, 33
Unused Oil (100 μℓ) + S9	40, 41
Used Oil (10 μℓ)	53, 43
Used Oil (10 μℓ) + S9	612, 637
Used Oil (20 μℓ) + S9	803, 845

Samples of used and unused (same brand) were extracted with DMSO in a 1:1 ratio, and the polycyclic aromatic fraction obtained was used directly in the Ames assay with Salmonella strain TA 98. Duplicate plate counts for a typical experiment are presented.

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CHEMICAL CARCINOGENESIS IN FISH: INDUCTION OF HEPATIC DRUG METABOLIZING ENZYMES AND BACTERIAL MUTAGENESIS WITH POLYCYCLIC AROMATIC HYDROCARBONS (PAH)

by

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ABSTRACT

Channel catfish, *Ictalurus punctatus*, and rainbow trout, *Salmo gairdeneri*, were exposed to the PAHs benzo(a)pyrene (BaP) and 3-methylcholanthrene (3-MC) and to polychlorinated biphenyls (PCBs) (channel Catfish). Livers were studied by biochemical and morphological methods. Fish injected once daily for a 7-day period with 50 mg PCBs/kg b.w. showed increased levels of cytochromes P-450 and b_5, and increased activity of NADPH cytochrome c reductase. Longer exposure (21 days) caused greater increase in the above and increased the activity of aminopyrine demethylase. Catfish exposed to BaP or 3-MC by i.p. injection or gastric intubation showed increased liver arylhydrocarbon hydroxylase (AHH) activity. Maximum induction (8-fold) followed 3 treatments totaling 75 mg 3-MC/kg b.w. Livers of trout actuely exposed to 3-MC showed a 3-fold increase in cytochrome P-450 and a 2-fold increase in NADPH cytochrome c reductase activity. Aminopyrine demethylase activity was also enhanced. Increased enzyme activity correlated with increased smooth endoplasmic reticulum of hepatocytes. Appropriate liver fractions from noninduced channel catfish and from 5-day induced (Aroclor 1254) rats were compared for ability to convert BaP to a mutation inducing compound in a bacterial mutagenesis assay. The number of revertants in both systems was nearly identical. Data suggest suitability of fish for carcinogen bioassay using PAH.

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INTRODUCTION

The aquatic environment ultimately becomes exposed to virtually every pollutant entering the biosphere (Revelle, 1968) and neoplasms have been reported within nearly all organic systems of feral fishes (Mawdesley-Thomas, 1975; Wellings, 1969). Thus, analysis of tumor incidence within fish populations might serve as a bioindicator of carcinogens in the aquatic environment. Some application of this approach has been made (Stitch and Acton, 1976). However, as Stewart (1977) has suggested, controlled laboratory exposure of different fish species to known carcinogens is needed to provide information concerning suitable species for bioassay and for those conditions of exposure necessary for tumor production. Such data would form a more precise basis for subsequent environmental monitoring of aquatic carcinogens.

Except for the aflatoxin-induced liver neoplasm of rainbow trout, in which data on metabolism of the carcinogen, histogenesis of the lesion, co-carcinogenic and synergistic factors, and metastasis of the tumor have been reported (Sinnhuber et al., 1977), limited data on laboratory chemical carcinogenesis studies are available for fish. The objective of our research group has been to test the feasibility of using fish as a monitor of aquatic carcinogens. To establish this system we used a correlated morphological/biochemical approach to analyze specific steps in the carcinogenic process, i.e., induction of drug metabolizing enzymes, activation of procarcinogens to mutagens, development of cellular culture systems for carcinogen binding studies, and long-term exposure to various carcinogens for the purpose of producing tumors. At each step, careful comparison between fish and mammals, primarily the rat, was undertaken to more carefully characterize the data in fish.

The polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental pollutants, some of which have proven carcinogenic activity (Saffiotti et al., 1968; Albert, 1976; Kraybill, 1976). PAH enrichment of sediments in lake and ocean has recently been linked to the utilization of coal (Muller et al., 1977) and petroleum (Hites et al., 1977). From the mammalian literature, it is known that the microsomal metabolism of PAH is a necessary prerequisite for binding of these components to cellular macromolecules (Harris, 1976). Once activation of PAH has taken place and binding to cell macromolecules including DNA has been demonstrated, mutations occur in bacterial mutagenesis systems (Ames et al., 1975). PAH have been shown to be metabolized by fish (Pedersen and Hershberger, 1974; Ahokas et al., 1977). This paper relates our findings on the induction of the fish microsomal mixed-function oxidative system (MFOS) by the PAH benzo(a)pyrene and 3-methylcholanthrene. The data for PAH are compared to the data for induction using the PCB mixture Aroclor 1254. Morphologic studies are correlated to biochemical findings and compare the effects by the inducing agents PCB and 3-methycholanthrene. In addition, our data comparing the fish MFOS with that of the rat in the Ames (Ames et al., 1975) mutagenic system, using benzo(a)pyrene (BaP), are presented.

MATERIALS AND METHODS

Animals

Channel catfish of both sexes, weighing 100 to 150 g were obtained from a local hatchery. Rainbow trout of similar weight were donated by the State of West Virginia Department of Natural Resources. Fish were acclimated and maintained in 100-gal living stream aquaria (Frigid Units, Inc.) and fed a pelleted ration (Purina trout chow) daily throughout the study.

At the start of the experiment, random fish were transferred to 20-gal tanks with aerated water at temperature of 12 to 15° C; 25% of the water from control and treated tanks was changed daily.

Treatment of Animals

Fish were exposed to either BaP, 3-MC, or PCB in corn oil via intraperitoneal injection or gastric intubation (Table 1). Control fish received identical treatment with corn oil only.

TABLE 1. SUMMARY OF ANIMAL TEATMENTS

Fish	Compound	Number of Fish	Treatment	Administration	Time of sacrifice (post-exposure)
Channel Catfish	PCB	15	50 mg/kg b.w. 6 daily doses	i.p.*	24 hours
	PCB	5	1000 mg/kg b.w. single dose	g.i.**	21 days
	BP	4	100 mg/kg b.w. single dose	i.p.	48 hours
	BP	4	25 mg/kg b.w. 6 daily doses	i.p.	24 hours
	3-MC	5	20 mg/kg b.w.	i.p.	24 hours
Rainbow trout	3-MC	12	50 mg/kg b.w. 4 daily doses	i.p.	24 hours

* intraperitoneal injection
** gastric insertion

Preparation of Microsomal Fractions

Catfish were killed by severing the spinal cord and the livers were rapidly removed, weighed, and placed in 0.5M Tris-1.5% KCl buffer, pH 7.4, on ice. Minced tissues were rinsed repeatedly to remove blood, and placed in fresh buffer. Trout were anesthetized in a 1:4000 solution of tricaine methane sulfonate (MS-222) in water. Blood was removed from the livers by whole body perfusion (Hinton, 1975) with a fish ringer solution at 10° C. Following perfusion, livers were rapidly removed, blotted, weighed, and placed in ice cold 0.3M sucrose. A 25% homogenate was made by four complete passes of a glass Potter-Elvehjem tube and teflon pestle at 260 rpm.

Cells, nuclei, mitochondria, and lysosomes were removed by centrifugation at 9,000 x g for 15 min. In order to obtain a microsomal pellet, the resulting post mitochondrial supernatant (S9) was sedimented at 100,000 x g for 60 min at 0 to 4° C. The pellet was resuspended in 0.5M Tris-1.15% KCl (catfish) or 0.3M sucrose (trout) to a final concentration of 0.5 grams liver/$m\ell$ of microsomal suspension. Microsomal protein concentration was determined by the method of Lowry _et al_. (1951).

Enzyme Assays

Cytochromes P-450 and b_5 were determined according to the method of Omura and Sato (1964). In order to estimate the amount of hemoprotein present, extinction coeffients of 91 mm^{-1} cm^{-1} at 450 -490 nm (P-450) and 170 mM^{-1} cm^{-1} at 424 -409 nm (b_5) were used. Aminopyrine demethylation was determined according to the method of Gnosspelius _et al_. (1969). The amount of formalydehyde formed was determined according to the procedure of Nash (1953). NADPH cyt-c reductase was determined by the method of Dallner _et al_. (1966), using an extinction coefficient of 19.1 mM^{-1} cm^{-1} at 550 nm for reduced cytochrome c. The previously determined optimal incubation temperature for fish microsomal assays (27 to 29° C) was used. Aryl hydrocarbon hydroxylase (AHH) was determined according to the method of Nebert and Gelboin (1968). Determination of optimum procedures for this assay was performed and a temperature of 29° C, a pH of 7.5, an incubation time of 20 min, and a microsomal protein concentration between 0.1 and 0.2 mg were used. The AHH activity was expressed as nanomoles of 3-OH BaP formed per 20 min per mg of microsomal protein.

AMES Mutagenic Bioassay

Liver homogenates (25%) from control channel catfish and Aroclor 1254 induced rats were centrifuged at 9,000 x g for 15 min. The S9 fractions were used as the microsomal source for the bioassay. Specially constructed mutants of _Salmonella typhimurium_ were obtained from Dr. Bruce Ames (University of California). Varying concentrations of BaP were added to varying amounts of S9 from either catfish or rat. These mixtures were incubated with one of the five modified strains of _Salmonella typhimurium_ according to Ames _et al_. (1975) and McCann and Ames (1976). The number of revertants were counted and means were expressed per mg S9 protein.

Electron Microscopy

Pieces of liver from control and treated fish were minced in 4% formaldehyde-1% glutaraldehyde in phosphate buffer, pH (Mawdesley-Thomas, 1975), for 24 hr. The tissue was post-fixed in 1% OsO_4, dehydrated in graded ethanol solutions, and embedded in Epon (Luft, 1961). Semi-thin sections (0.5-1μm) were stained with toluidine blue (Trump et al., 1961) and viewed under a light microscope. These sections were used as a histologic survey to determine lobular zones. Thin sections were stained with uranyl acetate and lead citrate and viewed under an AEl-6B or a Jeol 100B electron microscope.

RESULTS

MFOS Activity

The data on the MFOS response to PCB were reported in previous papers (Klaunig et al., 1978; Lipsky et al., 1978). Table 2 summarizes these results as % of controls. Acute PCB exposure caused a moderate induction of MFOS enzymes. Subacute PCB exposure (21 days) resulted in a significant increase in induction of MFOS activity over both control and acute PCB treatment. Both cytochrome P-450 and aminopyrine demethylase were increased three fold over control levels. Induction of aryl hydrocarbon hydroxylase (AHH) by two PAHs is shown in Table 2. The data are also presented in Table 2. 3-MC caused a 10-fold increase in AHH activity, while g.i. and i.p. exposure to BaP caused a 4- to 5-fold induction of hepatic AHH. Table 3 summarizes the response of the rainbow trout hepatic MFOS to acute 3-MC exposure. Cytochrome P-450 was induced to 331% of control amounts. NADPH-cyt c reductase was also induced to over two times the control activity. The largest degree of induction occured in AHH which was elevated to almost 14 times over the control activity.

In all of these studies there was little to no effect on the liver to body weight ratios or amount of microsomal protein.

Electron Microscopy

The ultrastructure of control channel catfish liver (Figure 1) was identical to that previously reported (Hinton and Pool, 1976). The acute and subacute effects of PCB on hepatocyte morphology were previously reported by this laboratory (Klaunig et al., 1979; Lipsky et al., 1978). In summary, acute PCB exposure resulted in proliferation of rough endoplasmic reticulum (RER) and formation of vesicular profiles. Little smooth endoplasmic reticulum (SER) response was seen in the acute study. However, subacute PCB exposure caused extensive SER proliferation in the form of vesicles and membrane whorls (Figure 2). Increased lipid, in the form of cytoplasmic vacuoles and as rounded aggregates inside membranes of endoplasmic reticulum (Figure 2), occurred in all affected hepatocytes. Control rainbow trout hepatic morphology was identical to that previously reported by Scarpelli (1976). Acute 3-MC treatment caused changes similar to that seen in channel catfish after acute exposure to PCB. An apparent

TABLE 2. SUMMARY OF INDUCTION STUDIES ON CATFISH HEPATIC MFOS COMPONENTS

	P-450	b_5	NADPH-cytc Reductase	Aminopyrine Demethylase	AHH
1. PCB, 7 days*	132%	296%	136%	N.C.	----
2. PCB, 21 days+	294%	223%	156%	362%	----
3. 3-MC**	----	----	----	----	1000%
4. BP i.p.**	----	----	----	----	380%
g.i.**	----	----	----	----	513%

All values = % of control
N.C. = No change
*from Lipsky *et al*. (1978)
+from Klaunig *et al*. (1978)
**new data this laboratory

TABLE 3. EFFECT OF ACUTE EXPOSURE TO 3-MC UPON MFOS OF TROUT LIVER

	P-450	Microsomal protein	NADPH cytc reductase	Aryl* hydrocarbon hydroxylase	Liver/body wt. x 10^{-2}
Control	0.175 ± .05	21.03 ± 10.2	11.24 ± 2.47	0.54 ± .06	1.40 ± .2
3-MC	0.579 ± .20	16.42 ± 1.03	25.61 ± 2.61	7.37 ± 1.38	1.29 ± .10
% induction	331%	No change	228%	1365%	No change

All values based upon 3 "pools" of 4 livers each ± standard deviation of the mean.
*values based upon 1 pool of 4 livers in control and in treated fish.

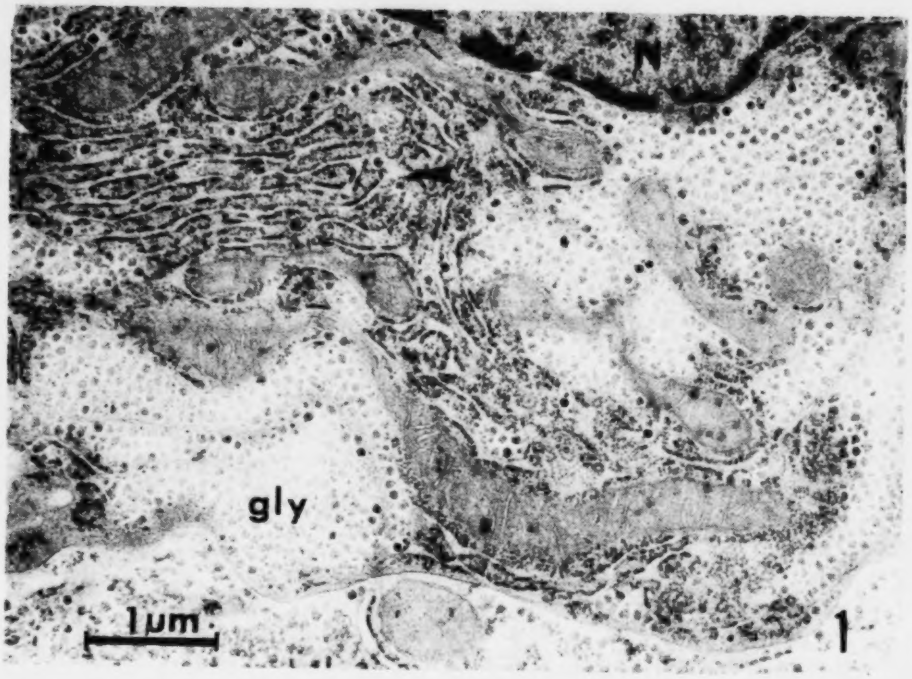

Figure 1. Portion of two hepatocytes from control channel catfish. Extensive glycogen deposits (gly) occupy large portions of cytoplasm. Mitochondria and parallel stacks of RER (arrow) exist together near nucleus (N) and in partitions between glycogen depot sites.

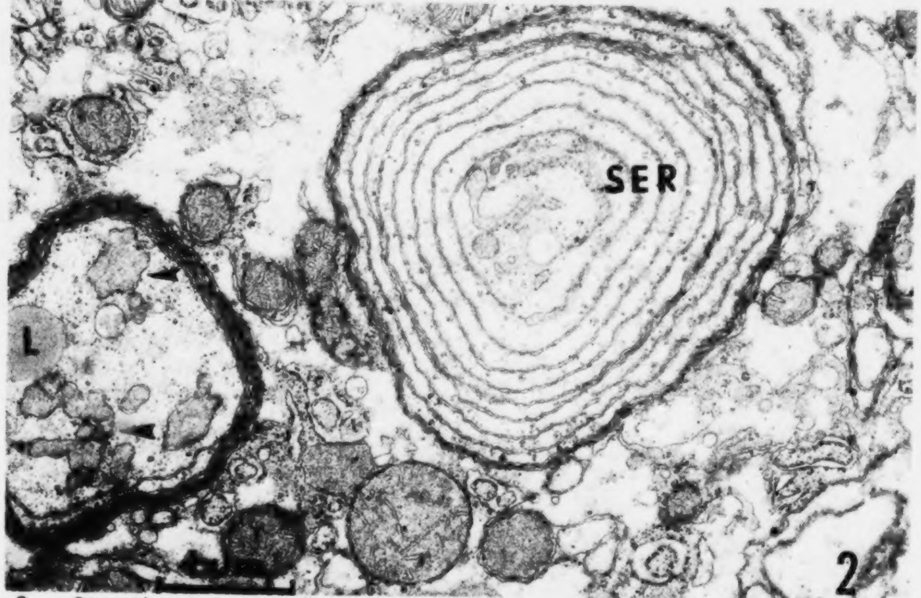

Figure 2. Smooth membrane "whorls" (SER) in catfish hepatocyte following subacute PCB exposure. Note lipid (L) droplet and membrane-bound lipid (arrows) in center of whorl.

increase in RER content and disruption of paralled cisternae, in some areas, with the appearance of vesiculated and dilated RER was noted (Figure 3). Dilated vesicles contained a material of low to moderate electron density. Scattered vesicular profiles of smooth membranes were also seen (Figure 3). An electron micrograph of a microsomal pellet from a control fish is shown in Figure 4. The pellet was composed of vesicular forms of rough and smooth-surfaced membranes. Some free ribosomes and glycogen particles were also seen. Pellets were free of mitochondria and lysosomes.

Bacterial Mutagenic Bioassay

The results of the Ames mutagenic bioassay are summarized in Table 4. The mean number of revertants per microgram of benzo(a)pyrene was calculated. BaP produced revertants in all four *Salmonella typhimurium* strains tested using rat or fish S9. Channel catfish S9 was approximately 74% and 39% as effective in producing revertants as rat S9 in the strains 1535 and 1537, respectively. However, in strains TA98 and TA100 S9 from channel catfish proved to be over 90% as effective as S9 from PCB-induced rat. Toxicity was noted at high benzo(a)pyrene concentrations in strain 1537 with both rat and fish.

DISCUSSION

Data presented here and in other papers in this symposium confirm earlier reports that the fish liver contains a microsomal mixed-function oxidase system, which is inducible by a variety of important environmental pollutants (Pohl *et al*., 1974; Bend *et al*., 1977a; 1977b) including the PAH, 3-MC, and BaP (Gruger *et al*., 1977; Statham *et al*., 1978). With respect to chemical carcinogenesis, perhaps the most important feature of the MFOS system is the induction of aryl hydrocarbon hydroxylase. This enzyme system has been shown to be responsible for converting procarcinogenic PAH into epoxides (Grover and Sims, 1968), more proximate carcinogenic forms (Harris, 1976). With all of the compounds that we have tested so far, biochemical induction of MFOS enzymes is accompanied by morphologic change, particularly in the endoplasm reticulum of hepatocytes (Klaunig *et al*., 1978; Lipsky *et al*, 1978). The acute response with PCB and 3-MC was identical. The paralled RER cisternae were maintained in some areas while dilated, vesicular profiles appeared in others. These data correlate well with the studies by Scarpelli (1976), using aflatoxin and with changes reported in mammalian liver (Smuckler and Arcosoy, 1969). The next step in our investigation, following the demonstration of induction of fish MFOS enzymes by PAH, was to determine whether PAH metabolites would cause mutations in a bacterial mutagenesis test. Since the bacterial screen utilizilng microsomal frac- tions from fish has been done in only a few labs (Ahokas *et al*., 1977; Stott and Sinnhuber, 1978), we ran parallel tests with rat S9 fractions, a system which has been studied in more detail (Ames *et al*., 1975). The fish microsomal fraction converted BaP into metabolites, which subsequently caused mutations in the bacterial tester strains. The number of mutants was nearly equal to those caused by similar fractions from the rat. These data are even stronger when it is noted that

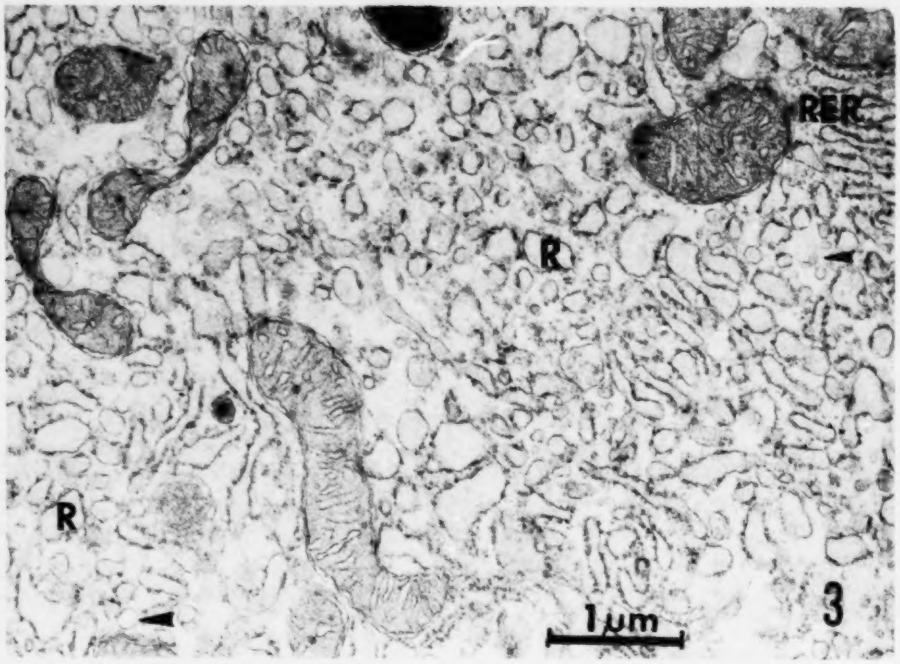

Figure 3. Portion of rainbow trout hepatocyte following acute 3-MC treatment. Extensive dilated RER vesciles (R) fill large area of cytoplasm. Parallel cisternae (RER) remain in some areas. Smooth surfaced vesciles are also apparent (arrows).

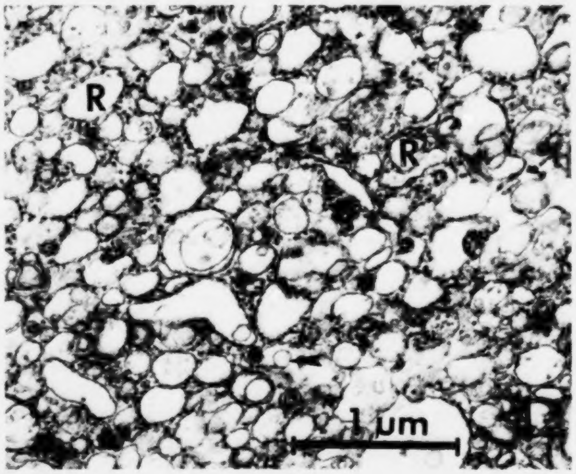

Figure 4. Typical microsomal pellet from control fish liver. Note both rough (R) and smooth (arrow) surfaced membranes.

TABLE 4. COMPARISON OF FISH AND RAT USING BaP IN AMES MUTAGENIC BIOASSAY

Salmonella Strain	S9 source*	Micrograms (μq) BaP							Mean
		0	5	10	15	20	25	30	
TA 98	Rat	1.6	24.5	50.0	63.3	66.7	85.2	129.0	3.35
	Fish	1.7	17.9	43.8	60.9	65.7	78.5	121.8	3.60
TA 100	Rat	7.7	20.6	25.8	39.8	54.6	74.0	87.8	2.44
	Fish	9.8	23.3	27.6	46.2	51.9	60.9	85.8	2.25
1537	Rat	0.4	8.6	12.7	11.9	15.5	- 0 -	- 0 -	1.92
	Fish	0.5	2.5	12.2	11.8	12.4	20.1	- 0 -	0.76
1535	Rat	1.1	10.2	25.8	39.8	58.2	82.7	104.1	3.20
	Fish	1.3	6.2	18.8	30.7	46.2	63.9	91.1	2.38

* 50 μℓ of S9 per plate.
All values represent the mean number of revertants per milligram S9 protein per plate (minus background).

the rat was induced with Aroclor 1254 for 5 days prior to the preparation of the S9 fractions. Thus, we can state that fish not only have an MFOS system containing enmzymes similar to those in the rodent liver but also have a capacity for making mutagenic metabolites of procarcinogens. Subsequent studies are needed to determine whether MFOS induction and metabolic activation of PAH will actually lead to tumor formation in fish. In this regard, it is interesting to note that 3-MC and BaP caused epitheliomas when painted onto skin of fish (Ermer, 1970). The wide geographic distribution of PAH and their induction of carcinogenesis-associated enzymes makes this system warrant further investigation. In our laboratory, we are now ready to compare changes seen in human cells with those in fish and rat cells and to continue long-term feeding protocols in an effort to induce tumors *in vivo*. It is through the use of these *in vitro* assays that interspecies comparisons and extrapolation to man are possible. These studies are necessary (Stitch and Acton, 1976) so that some assessment of the applicability of findings in fish to potential human health problems can be made.

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INDUCTION OF BENZO(A)PYRENE MONOOXYGENASE IN FISH AFTER I.P. APPLICATION OF WATER HEXANE EXTRACT -- A PRESCREEN TOOL FOR DETECTION OF XENOBIOTICS

by

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ABSTRACT

Carps, Cyprinus carpio, benzo(a)pyrene monooxygenase (BaPMO) is inducted after intraperitoneal (i.p.) application of benzopyrene, methylcholanthrene, benzoanthracene, aflatoxin, or Libyan crude oil. The kinetics of BaPMO induction in experimental fish have been studied at various concentrations and at various exposures. At a fixed time of exposure of 48 hr, the dose-response was established after i.p. application of a series of different concentrations of crude oil or benzo(a)pyrene dissolved in and extracted from charcoal-treated seawater samples. The induction-response could be traced in 1000 mℓ of seawater extraction to concentrations of 0.5 parts per million (ppm) of crude oil. This range of sensitivity lies well within the range of naturally occurring concentrations in the marine environment and is applicable in field monitoring of hydrocarbons.

INTRODUCTION

Benzo(a)pyrene monooxygenase (BaPMO) is induced in fish exposed to petroleum (Payne and Penrose, 1975; Ahokas et al., 1976; Kurelec et al., 1976; Gruger et al., 1977). BaPMO measurements in local fish appear to be a practical biological indicator for monitoring marine petroleum pollution (Payne, 1976), enabling the identification of polluted marine areas and a follow-up study of the consequences of an oil-pollution incident by measuring the increased BaPMO levels in the livers of the local Blenniidae (Kurelec et al., 1977). It seems that presence of inducible microsomal mixed function oxydases (MFO) activity in fish (Bend et al., 1977) could serve as a useful biochemical diagnostic tool for environmental monitoring and, at the same time, as a relevant parameter in evaluation of the effects of acute or chronic pollution at a given site. However, from the ecological and the environmental assessment point of view, it would be highly desirable to detect xenobiotics present in water by means of this early and relevant biochemical parameter. Such a method would fill the gap which usually exist between the estimated concentration of xenobiotics in water and corresponding biological effects. At the same time, this method could be used as a prescreen tool to design cost-effective xenobiotic monitoring programs using expensive analytical chemical methods.

We studied the consequence of intraperitoneal (i.p.) application of extracts from polluted water on the liver BaPMO in young carp. The inducibility and the basic characteristics of BaPMO in carp and the dose-response of BaPMO after the i.p. application of polluted water extracts are demonstrated in our first attempts to apply this method for detection of xenobiotica in the marine environment.

MATERIAL AND METHODS

Chemicals

Benzo(a)pyrene (BaP) was from Roth, Karlsruhe, Germany; 9,10 dimethyl 1,2-benzanthracene and 20-methylcholanthrene were from Calbiochem, Lucerne, Switzerland; NADPH from Serva, Heidelberg, Germany; hexane, fluorescent grade, was from Merck, Darmstadt, Germany, and corn oil, commercial grade, was from Oil Factory, Zagreb, Yugoslavia. "Sarir", Lybian crude oil, was a gift from INA-Zagreb. Its paraffin content was 14 to 19% and the density at 15° C was 0.843 g/cm^3. All other chemicals were of analytical grade.

Seawater Samples

Samples were collected seasonally at 8 stations in the Rijeka Bay and 3 stations in the vicinity of Rovinj, within the framework of the project "Ecological Study of Rijeka Bay" (Project Rijeka) and "Ecological Study of Rovinj Area" (Project Rovinj), respectively. Samples were collected with a sampler at a depth of 1 m. Experimentally polluted seawater samples were prepared with charcoal treated seawater as the base. Quantities of Libyan crude oil were dispersed in 50 mℓ of charcoal treated seawater with Ultra-Turrax (Janke and Kunkel, Staufen, Germany) and then mixed with

950 mℓ of charcoal treated seawater; 1-ℓ samples of test seawater or of experimentally polluted seawater were extracted with hexane and the extract prepared for i.p. application as described previously (Kurelec *et al.*, 1979). Aliquots of these extracts were screened for their content of extractable substances by measuring their fluorescence at an activation wavelength of 313 nm and an emission wavelength of 360 nm in a Zeiss PMO 3 spectrophotofluorimeter calibrated with hexane on 1000 fluorescence units (f.u.).

Animals

One-year-old specimens of artificially hatched carp, *Cyprinus carpio*, weighing 8 to 12 g were adapted for 1 month in 200-ℓ basins, with 150 ℓ of dechlorinated, well-areated water, at a density of 400 specimens/m^3 at a flow of five total changes per day at 14° C and then used in the experiments. Hexane extracts, dissolved in 0.1 mℓ of corn oil, were injected i.p. to carp from 0800 to 1000 hr. Animals were given no food during the experimental period.

Homogenate Preparation

Subcellular fractions and BaPMO assay with protein measurements were performed as previously described (Kurelec *et al.*, 1977).

RESULTS AND DISCUSSION

Some Properties of BaPMO in Carp

The induction of BaPMO was measured in carp subsequent to the i.p. application of a single dose of different substances. The results of the BPMO activity measurements after various exposure times are presented in Table 1. In these experiments, the postmitochondrial liver fraction of a carp which had been induced by a single dose of the test substance was estimated at a optimal pH (7.4) and at a temperature of 29° C. The velocity of benzo(a)pyrene hydroxylation was proportional to the amount of postmitochondrial fraction from 25 μℓ to 150 μℓ, or in the range from 0.04 to 0.23 mg of proteins per sample.

Time Course of BaPMO Induction

A group of carp was treated i.p. with a single dose of benzo(a)pyrene dissolved in 0.1 mℓ of corn oil at a dose of 10 mg/kg. During the first 24 hr, 4 specimens were sacrificed every 2 hr. At 36, 48, 72, and 96 hr, after injection additional fish were killed. The BaPMO activity was quickly estimated and the rise in the activity was plotted (Figure 1).

Induction of BaPMO with Extracts of Seawater Experimentally Polluted with Crude Oil

A series of 12 carp were treated i.p. by injection of 0.75, 1.5, and 15 mg of crude Libyan oil dissolved in 0.1 mℓ of corn oil. Another group

TABLE 1. INDUCTION OF BaPMO ACTIVITY IN CARP AFTER I.P. APPLICATION OF SINGLE TREATMENT WITH DIFFERENT SUBSTANCES

Substance	Dose in mg/kg body weight	Exposure time in hr	BaPMO activity in a. u.	
Benzo(a) pyrene	5-7	24	171.1 ± 70.8	(5)
	40		2600 ± 1870	(2)
	4-5	48	1920 ± 20.2	(2)
	0.15	72	73 ± 56	(2)
	1.0	72	296 ± 145.6	(2)
	3.0	72	820	(1)
	6-7	72	2463 ± 886.4	(3)
	5	120	4540	(1)
20-Methylcholanthrene	2	24	1749	(1)
	6	24	2143 ± 550	(2)
	8-11	24	2035 ± 1065	(3)
	5	72	1398	(1)
	7	72	2549	(1)
	4-7	120	1132 ± 60.8	(2)
9,10 Dimethyl 1-2 benzanthracene	5	24	1100	(1)
	4	120	846	(1)
Phenobarbital	100	72	18.9 ± 3.6	(2)
Control			23.8 ± 19.9	(7)

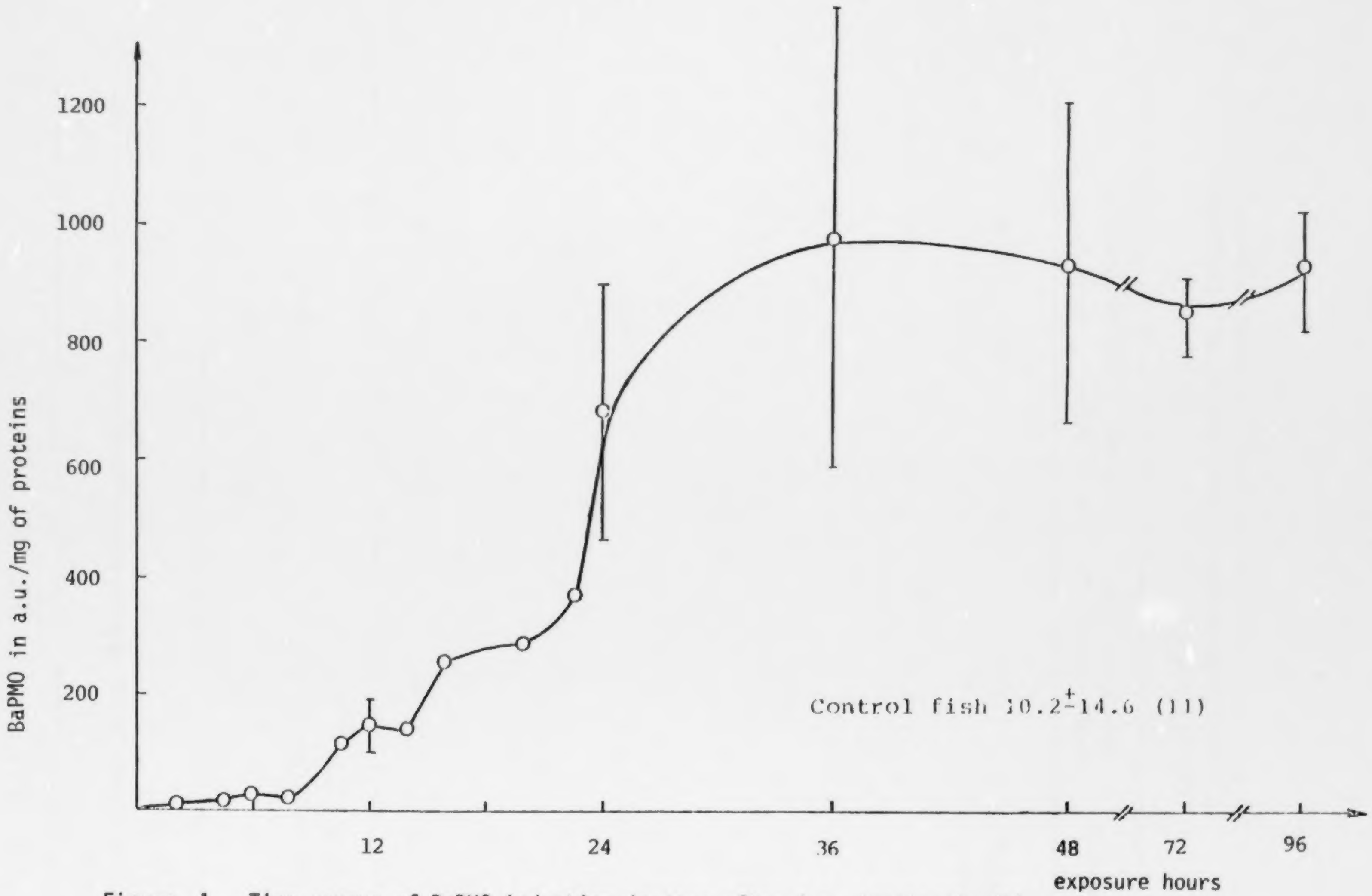

Figure 1. Time course of BaPMO induction in carp after i.p. treatment with a single dose 10/mg/kg benzo(a)pyrene.

was injected with the hexane-extracted material from 1-ℓ charcoal-treated seawater samples to which 0.75, 1.5, and 15 mg of crude oil were added. All fish were exposed for 48 hr. The dose-response of BaPMO-induced activity is shown in Table 2. Prior to evaporation, fluorescence of hexane extracts of the experimentally polluted seawater were measured. The results are plotted in Figure 2.

Hexane extracts of seawater samples collected at 8 stations from the Project Rijeka revealed of 232.7±315.6 fluorescence units, which is equivalent to 8.0±10.9 μg/ℓ crude oil. The range of fluorescence units in 13 samples lies between 50 and 1022 f.u. (or the equivalent of 1.72 to 35.2 μg/ℓ crude oil). As will be shown, these fluorescent materials revealed higher biological activity than the material of the crude oil-origin which had a 20-fold higher fluorescence.

Field Observations

In September and December 1977, and in March and June 1978, we collected samples of seawater within the work on Rijeka Project at 8 stations (Figure 3). The samples (1 m depth) were extracted expeditiously with hexane and the amount of fluorescent material estimated. BaPMO tests were accomplished within a week. The results of a March 1977 excursion, which was chosen as a representative out of four similar results obtained in other excursions, are presented in Table 3. Although the biological activities of these samples were low, the discrepancy between the fluorescence value and the BaPMO induction are obvious. Station A in Table 3 was a coastal site where we estimated the seasonal BaPMO level in a territorial fish, *Blennius pavo*. There, 500 m from the petroleum processing discharge outlet, these fish were highly induced relative to fish in five other sites (see Figure 3). Seawater samples taken there contained a large amount of extractable material that gave a high BaPMO induction.

On March 29, 1978, samples of seawater collected at three stations from the Project Rovinj (Figure 4) revealed an interesting phenomenon: samples from sites R4 and R5, containing the "usual" concentration of hexane extractable material (as estimated by fluorescence units) do contain substances that are very biologically active substances (see Table 4).

It seems likely that this active material also caused extreme BaPMO induction in the population of *Mugil cephalus* that existed for a long period in the vicinity of a fish-cannery discharge. We have shown that hexane-extracted material from seawater samples taken at the border of the "mixing zone" of this pollution source possessed premutagens and/or precarcinogens, since they caused an increase of the number of *his+* revertants of *Salmonella typhymurium* strain T 100 after activation with postmitochondrial fraction of liver homogenates from induced Mugil specimens (Kurelec *et al*., 1979).

TABLE 2. INDUCTION OF BaPMO WITH EXTRACTS OF SEAWATER EXPERIMENTALLY POLLUTED WITH CRUDE OIL*

	Experiment No	Dose in mg of crude oil/kg body weight			
		0	75	150	1500
Crude oil	1	23 8 ± 19.9 (7)	21 ± 2.8 (2)	50.5 ± 17.7 (2)	124 (1)
Hexane extracts	1	23.8 ± 19.9 (7)	38 ± 11 (5)	50 ± 23.4 (6)	124.8 ± 36.7 (4)
	2	44.6 ± 9.9 (10)	63.8 ± 15.1 (4)	61.8 ± 12.8 (4)	156.8 ± 75.7 (4)

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* BaPMO ACTIVITIES ARE EXPRESSED IN UNITS/mg OF PROTEIN ± STANDARD DEVIATION. NUMBER OF SPECIMENS USED IN EXPERIMENTS ARE IN PARENTHESIS.

TABLE 3. BaPMO INDUCTION IN CARP TREATED I.P. WITH HEXANE EXTRACTS OF SEAWATER SAMPLES FROM RIJEKA BAY

March 8, 1978 Station	Fluorescence units of hexane extracts	BaPMO induction in a.u./mg protein
1	1252	22
12	1220	28
9a	5088	14
19	1236	5
7	1771	34
20a	1348	17
11	1200	30
8a	1410	8
A	8240	925

TABLE 4. INDUCTION IN CARP TREATED I.P. WITH HEXANE EXTRACTS OF SEAWATER SAMPLES COLLECTED IN THE VICINITY OF ROVINJ

March 29, 1978 Station	Fluorescence units of hexane extracts	BaPMO induction in a.u./mg protein
R4	1280	706
R5	1140	302
R6	2230	34

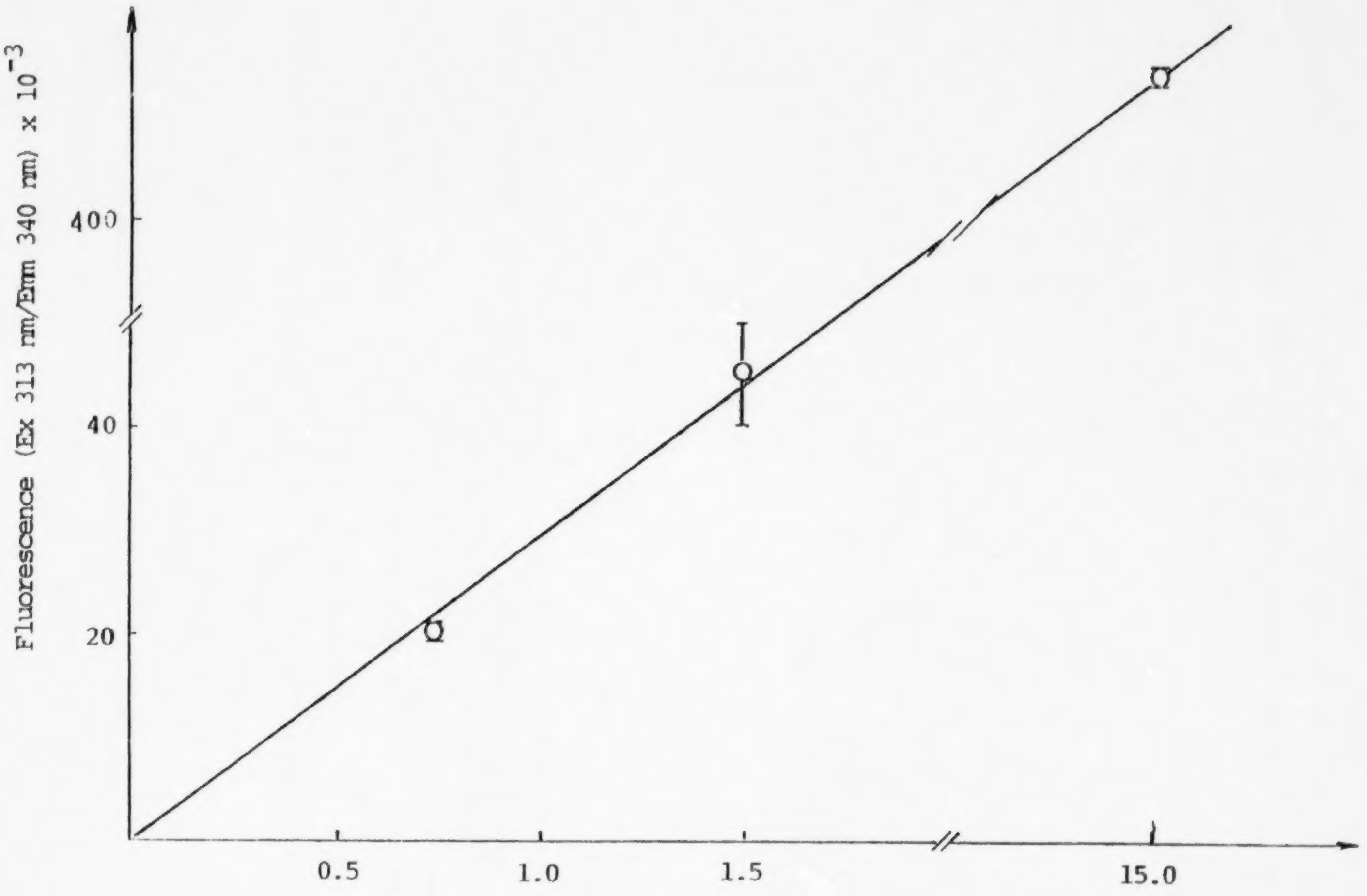

Figure 2. The fluorescence of hexane extracts of the seawater treated with 0.75, 1.5, and 15 mg/ℓ crude oil.

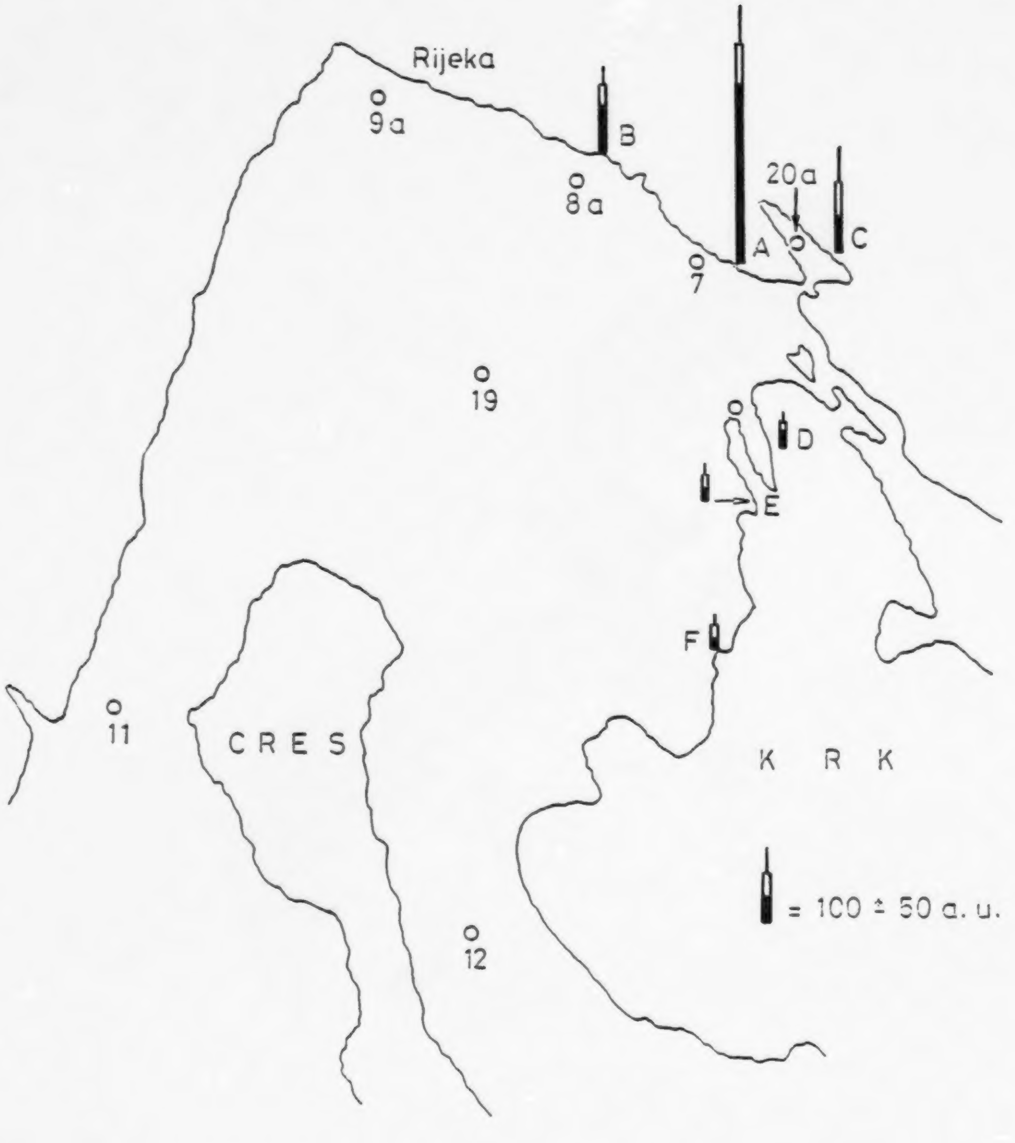
Rijeka
9a
B
8a
20a
A
C
7
19
D
E
F
11
CRES
KRK
12
= 100 ± 50 a. u.

Figure 3. Sampling sites in the Rijeka Bay where samples of seawater (numbers) and specimens of Blennius pavo were collected (capitals).

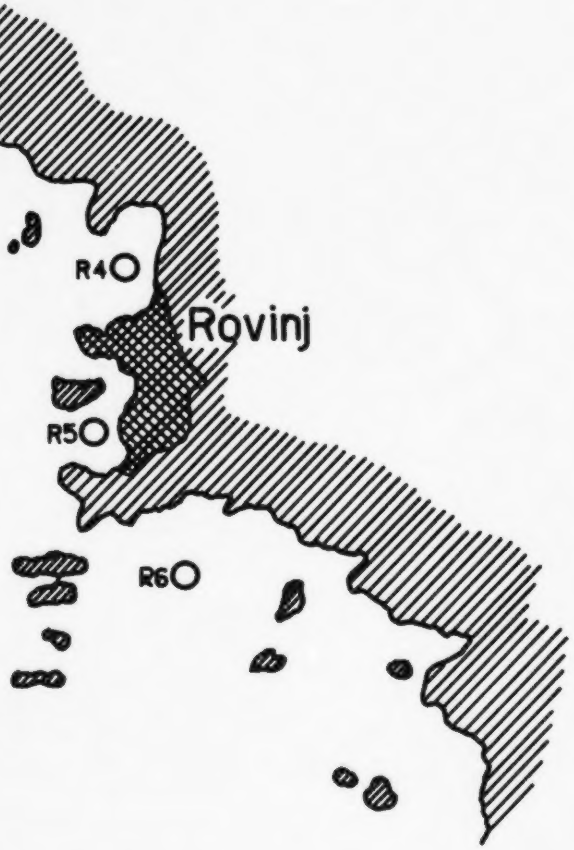
R4
Rovinj
R5
R6

Figure 4. Sampling sites in the vicinity of Rovinj.

SUMMARY

In some cases, biological responses were concentration-dependent. In others, fluorescent material was not biologically active, or considerable biological activity was not correlated with the corresponding fluorescence.

These observations indicate that BaPMO induction could distinguish two types of hexane extractable fluorescent material: biologically active substances and biologically inactive ones. In addition, this system monitors hexane extractable material that does not fluoresce as expected, i.e., it detects xenobiotics where fluorescence monitoring methods fail to signal their presence. In the present work, BaPMO was shown to be induced by known carcinogens, crude oil, complex petroleum processing discharges, domestic sewage, and discharges from a fish cannery. This demonstrates that BaPMO induction in fish after i.p. application of hexane extractable fractions could be used in a survey for xenobiotics in the marine environment. A special value would represent, as in our field observations, the use of the BaPMO induction test as a prescreen tool for developing an appropriate cost-effective analytical method that should (or should not) be used in specific sites. The method described here brings a new quality for the application of MFO induction monitoring in environmental studies: it sensitively detects xenobiotics in any given sample of water. I.p. induction, was strongly correlated with naturally occuring induction in fish living in the corresponding water. Therefore, substances that induce BaPMO after i.p. treatment apparantly would induce fish living in polluted environment. In addition to the diagnostic value for one biochemical parameter, the BaPMO induction also has a predictive value in the assessment of the environmental hazard from toxic, mutagenic, or carcinogenic substances (Kurelec et al., 1979). BaPMO induction monitoring may allow us to distinguish environmentally hazardous substances from innocuous ones. This fact, therefore, could be a relevant basis for the development of regulatory criteria in water quality control. In our experiments, carp BaPMO was induced significantly only at crude oil concentrations above 1 mg/ℓ. These levels represent realistic environmental levels near petroleum processing discharges that are limited by U.S. regulations to an effluent level of less than 1.0 mg/ℓ freon extractables (D.J. Baumgartner, personal communication).

We intend to improve the method further by both technical and biological means. The latter include the use of either F_1-sister generation of fish and the use of monoclonal fish species (Hart et al., 1977), which will decrease background deviation and give more uniform induction-response. We hope to further increase the sensitivity of the method, but not circumvent the necessity of the development of regulatory criteria based on the rational use of early indicators for environmental contaminations by compounds that can be bioactivated to cytotoxins, mutagens, and carcinogens.

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IN VIVO AND IN VITRO STUDIES ON THE METABOLISM OF POLYCYCLIC AROMATIC HYDROCARBONS BY MARINE CRABS

by

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ABSTRACT

Polycyclic aromatic hydrocarbons were taken up from the food or water. The metabolism of these aromatics resulted in hydroxylation of the parent compound followed by excretion of the metabolites. *In vitro* assay for aryl hydrocarbon hydroxylase showed high activity in the stomach tissues of both male and female crabs. The green gland, an organ with functions similar to the vertebrate kidney, was high in activity only in the female crab. The aryl hydrocarbon hydroxylase activity in the green gland varied during the molt cycle with large increases after the final molt. These results suggest that the green gland may function as a regulator of hormone levels. The effects of various inhibitors, including detergents, phospholipase, cytochrome c, carbon monoxide, piperonyl butoxide, and benzoflavone indicated that aryl hydrocarbon hydroxylase system in crabs was composed of cytochrome P-450, phospholipid, and cytochrome P450 reductase.

INTRODUCTION

Effects of aromatic and other petroleum hydrocarbons on a wide variety of marine benthic crustaceans (e.g., shrimp, crabs, and lobster) have been investigated (Anderson *et al*., 1974; Atema and Stein, 1974; Caldwell *et al*., 1977; Eorns, 1977; Karinen and Rice, 1974). When oil spills reach intertidal areas, the crabs are among the most severely affected animals. In the San Francisco Bay spill and Buzzards Bay spill, many years were required before shore crabs, *Pachygrapsus*, or fiddler crab *Uca*, recolonized heavily oiled areas (Chan, 1975; Kreb and Burns, 1977). Crab larvae are susceptible to the water soluble fractions of oil, possibly due to frequent molts during this period of their life history (Mecklenburg *et al*., 1977; Katz, 1973).

Crabs can take up aromatic hydrocarbons from food or water. These hydrocarbons are oxidized to various products and the metabolites are later excreted in the urine or feces (Lee *et al*., 1976; Corner *et al*., 1973). This paper summarizes studies on the metabolism of PAH polycyclic aromatic hydrocarbons by anomuran and brachyuran crabs. The tissue and enzyme systems involved in these reactions are discussed.

Metabolism of Polycyclic Aromatic Hydrocarbons by Living Crabs

Exposure of blue crabs, *Callinectes sapidus*, to a variety of radiolabeled aromatic hydrocarbons including benzo(a)pyrene (BaP), fluorene, naphthalene, methylnaphthalene, and methylcholanthrene) in food or water resulted in metabolism of those compounds to various phenols, diols, and their conjugates (Lee *et al*., 1976). The hepatopancreas contained highly polar metabolites--predominantly diols and their conjugates, whereas the blood had phenols and diols. The build-up of metabolites in the hepatopancreas suggested that this organ was important in hydrocarbon metabolism. The green gland, which has excretory functions, had no hydrocarbons and all radioactivity was in the form of highly polar metabolites.

Corner and coworkers (Corner *et al*., 1973) exposed the spider crab, *Maia squinado*, to food containing naphthalene. After uptake the urine contained unchanged naphthalene, 1,2-dihydro-1,2-dihydroxynaphthalene, a glucoside of this compound, 1-naphthyl sulphate, and 1-naphthyl glucoside. The presence of glucosides and sulfate indicated that crabs have the required conjugating enzymes. In mammals glucuronic acid is the main glycosidic conjugate but in crustaceans and insects it appears that glucose serves as the glycoside (Corner *et al*., 1973; Kahn *et al*., 1974; Elmamlouk and Gessner, 1977).

In Vitro Metabolism of Polycyclic Aromatic Hydrocarbons by Crabs

Extensive studies have been conducted on the metabolism of aromatic hydrocarbons by microsomal preparations of mammalian livers (Houston, 1975). The initial reaction is an oxidation catalyzed by an oxygenase system to form arene oxides. These reactive arene oxides can be nonenzymatically hydrated to phenols, enzymatically hydrated to diols by an epoxide hydrase, or glutathione conjugates can be formed by the action of glutathione-S-transferase (Bend *et al*., 1976; Lu *et al*., 1976).

In an assay using formation of hydroxybenzo(a)pyrene from BaP, significant mixed function oxygenase (MFO) activity has been noted in the microsomes from the stomach and green gland of blue crabs, *Callinectes sapidus* (Singer and Lee, 1977). However, only very low activity was detected in the hepatopancreas. This low activity in the hepatopancreas was at least partly due to a MFO inhibitor in this tissue. A similar inhibitor has been reported in the hepatopancreas of lobster (James *et al*., 1977). Using the amount of aldrin epoxide formed for the assay, Burns (1976) reported MFO activity in the green gland of the fiddler crab, *Uca pugnax*. Low MFO activity has been detected in gonadal and gill tissues of the blue crab (Singer and Lee, 1977).

Characterization of the MFO system in the stomach of blue crabs indicated that all activity was in the microsomes and maximal enzyme activity was attained at 30° C and pH 7.5 in the presence of NADPH, oxygen and magnesium (Singer *et al*., 1978). The association of the enzyme activity with the microsomes (collected at 100,000 x g) indicated that bacteria were not responsible for the observed hydrocarbon degradation since bacteria were removed by the preliminary low speed centrifugation (800 x g).

The components of the MFO system in mammals are cytochrome P-450, NADPH cyctochrome P-450 reductase, and phospholipid (Lu *et al*., 1976). The MFO system in crab stomach appeared to be composed of similar components (Singer *et al*., 1978). P-450 inhibitors tested included SKF-525A at 10^{-4}M(48% inhibition), 10^{-6}M 7,8-naphthoflavone (84% inhibition), and 10^{-5}M piperonyl butoxide (70% inhibition). The presence of NADFPH cytochrome p-450 reductase activity was determined with cytochrome c as the electron recipient. MFO activity was inhibited when cytochrome c was added to the assay. The phospholipid requirement for crab stomach MFO was shown by the inhibition of MFO activity by detergents and phospholipase C.

The primary product of BaP metabolism by crab stomach microsomes was 3-hydroxybenzo(a)pyrene. This was shown by the ultraviolet fluorescence spectra of the isolated metabolites compared with the authenthic standards (Figures 1 and 2). BaP metabolite had a retention time identical to 3-hydroxybenzo(a)pyrene and a small peak tentatively identified as 9-hydroxybenzo(a)pyrene (Lee and Gonsoulin, 1978). In addition to BaP the crab stomach microsomal preparations were presented with four other polycyclic aromatic hydrocarbons, which included phenanthrene, chrysene, benz(a)anthracene and dimethylbenz(a)anthracene. The primary products of dimethylbenz(a)anthracene and benzo(a)pyrene metabolism were the phenolic metabolites while for the other hydrocarbons diol derivatives were predominant (Table 1).

No induction of MFO activity in stomach or green glands was observed when blue crabs were injected with either benz(a)anthracene or phenobarbital. Food contaminated with benz(a)anthracene was fed to crabs but resulted in no increase in stomach MFO activity. Fiddler crabs collected from a salt marsh contaminated with oil showed no difference in MFO activity compared with crabs from a clean area (Burns, 1976). In contrast, insects, fish and mammals all showed higher MFO activity after exposure to polycyclic aromatic hydrocarbons (Gelboin, 1972; Philpot *et al*., 1976; Payne and Penrose, 1975; Wilkinson and Brattsten, 1972).

Using ^{14}C-styrene oxide as the substrate, James and coworkers (1977) found high epoxide hydrase activity in microsomal preparations from the hepatopancreas of the rock crab, *Cancer ittorus*, and the blue crab, *Callinectes sapidus*. Hepatic glutathione-S-transferase activity was less in these crabs than epoxide hydrase activity whereas glutathione-S-transerase was higher in activity in mammals, suggesting that microsomal hydrases in crabs may be relatively more important for epoxide detoxification than in mammals. The presence of glucoside and sulfate conjugates of naphthalene metabolites in the urine of naphthalene exposed spider crabs

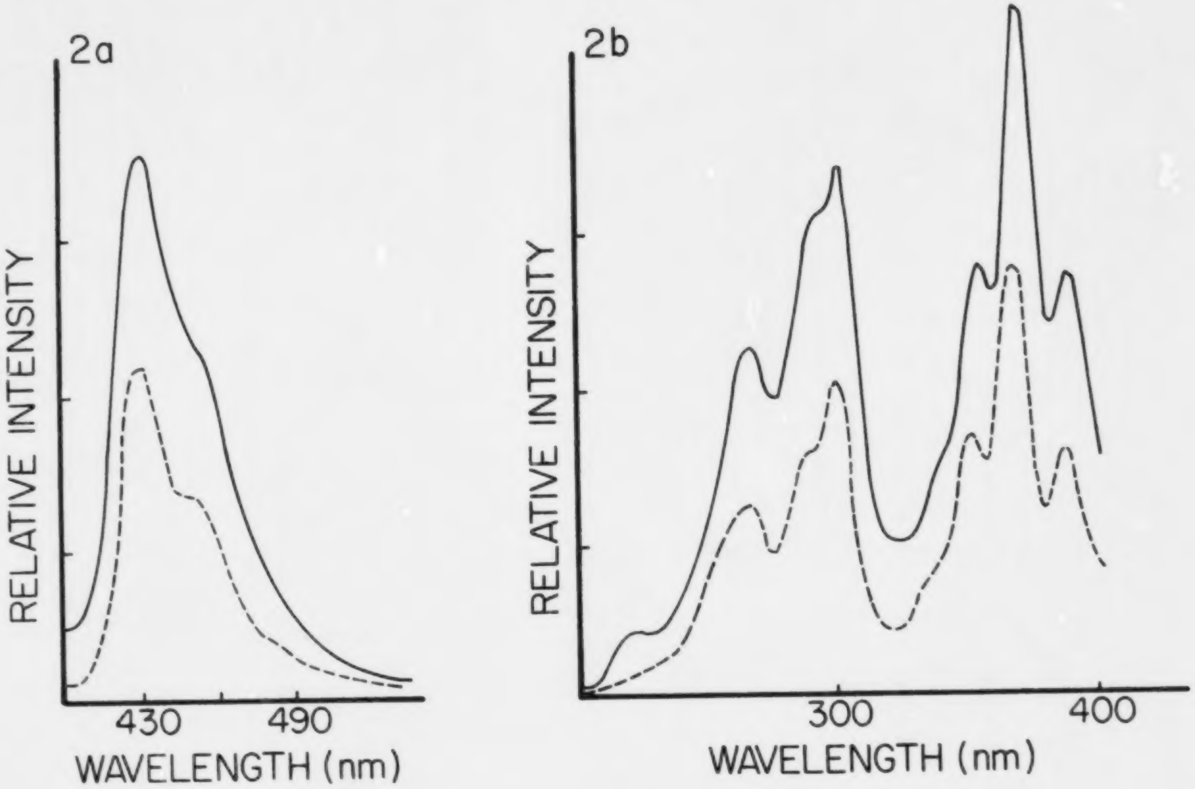

Figure 1. (a) Emission spectra of 3-hydroxbenzo(a)pyrene in ethanol isolated from in vitro assay (--------)of arylhdrocarbon hydroxylase and from an authentic standard (————). Excitation wavelength set at 374 nm. (b) Excitation spectra of 3-hydroxybenzo(a)pyrene in ethanol as in (a). Emission wavelength set is 430 nm (From Singer and Lee, 1978).

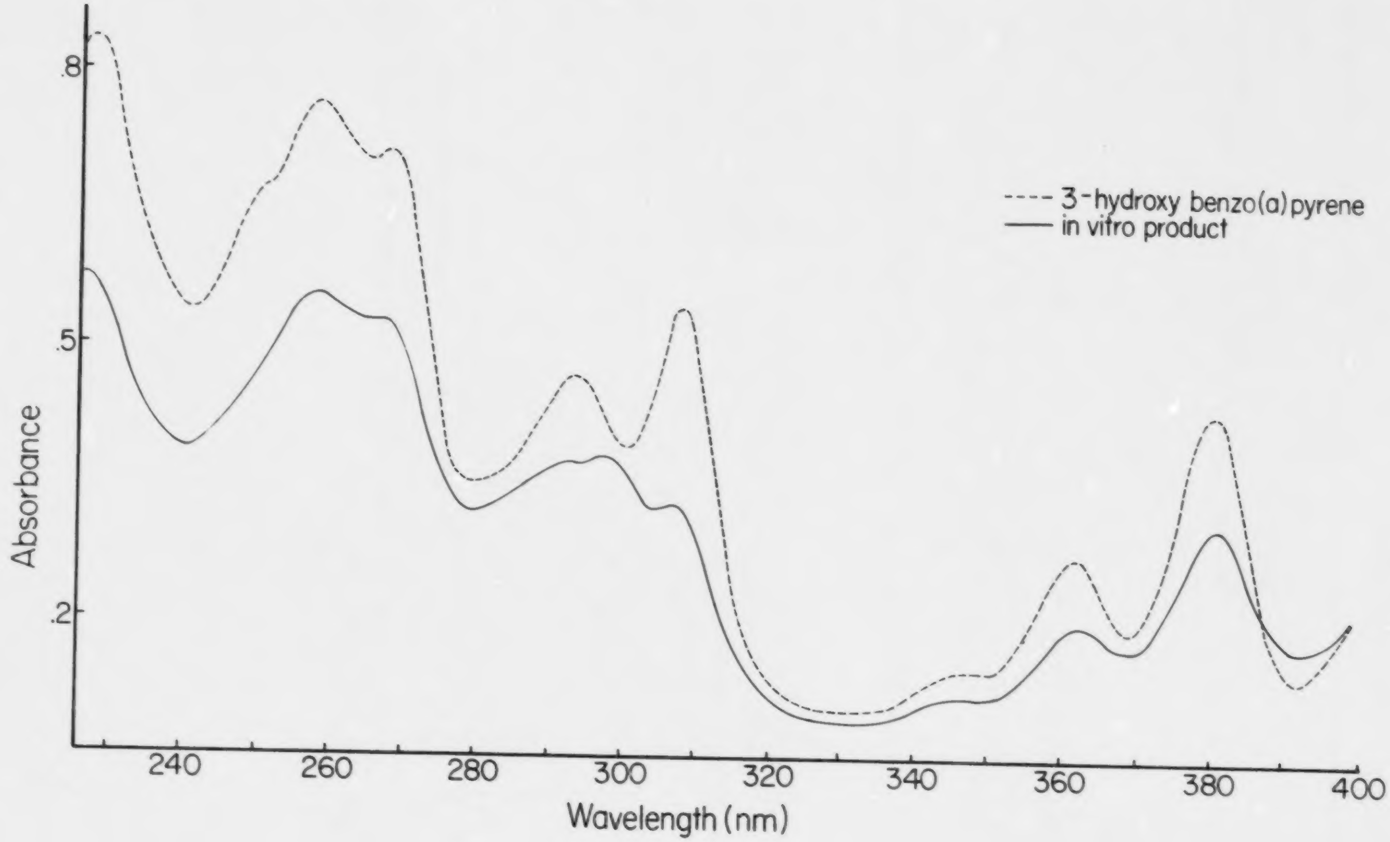

Figure 2. UV spectra of 3-hydroxybenzo(a)pyrene in ethanol isolated from *in vitro* assay (——) of arylhydrocarbon hydroxylase and from an authentic standard (-----) (from Singer and Lee, 1978).

TABLE 1. MODIFICATION *IN VITRO* OF VARIOUS ARYLHYDROCARBONS BY STOMACH MICROSOMES FROM BLUE CRAB*

Substrate	Phenols (pmole/hr)	Diols (pmole/hr)	Specific Activity (pmole/hr mg)
Phenanthrene	0	39	65
Benz(a)anthracene	10	20	50
Dimethylbenz(a)anthracene	187	64	410
Chrysene	7	16	38
Benzo(a)pyrene	88	4	154

* Incubation mixture had 1 mg microsomal protein, 0.6 μmoles $MgCl_2$, and 0.02 μmoles radiolabeled aromatic hydrocarbons. The compounds were (9-^{14}C) phenanthrene (11.3 μCi/μmole), (12-^{14}C)benz(a)anthracene (49 μCi/μmole), 7, 12-dimethyl(12-^{14}C)benz(a)anthracene (21 μCi/μmole), [5,6(11,12-^{14}C)] chrysene (6.3 μCi/μmole), and (7, 10-^{14}C)benzo(a)pyrene (51 μCi/mole). Assays were performed in triplicate and incubated for 60 min. The assay was terminated by adding 1 mℓ cold acetone and 3 mℓ hexane. The entire organic phase was evaporated under nitrogen gas and quantitatively spotted on precoated silica-gel thin-layer plates. The plates were developed in benzene: ethanol (9:1) and allowed to dry. Areas of the plates occupied by monohydroxylated (phenols) and dihydroxylated (diols) products corresponding to the R_f's of authentic standards 3-hydroxybenzo(a)pyrene and 5, 6-dihydroxybenz(a)anthracene were marked, scraped, and radioactivity determined in a liquid scintillation mixture. All other areas of the plates were pooled and determined for radioactivity in the same manner. Units are picomoles per hour (pmol/hr).

(Corner *et al.*, 1973) suggests the presence of UDP-glucose transferase and sulfate transferase in these crabs. Elmamlouk and Gessner (1977) showed glucosylation of p-nitrophenol by homogenates of hepatopancreas of the lobster, *Homarus americanus*.

Interactions of Mixed Function Oxygenase, Steroid Hormones, and Polycyclic Aromatic Hydrocarbons

The presence of MFO activity in the stomach of crabs suggests this organ is important in the metabolism of aromatic hydrocarbons encountered via the food. The role of the MFO system in the green gland is not clear since this organ is considered to have primarily excretory functions. Crabs exposed to hydrocarbon showed no buildup of hydrocarbons in the green gland and the presence of only polar metabolites as expected for an organ with excretory functions (Lee *et al.*, 1976). The MFO activity in the blue crab green gland changes during development (Figure 3) in a manner similar to that described for insects during their molting cycle (Perry and Buckner, 1970; Yu and Terriere, 1971). The changes in green gland MFO activity and levels of molting hormones can be correlated thus: molting in crustaceans is controlled by a group of steroid hormones called ecdysones produced by the y organs (Passano, 1960; Goad, 1976); during intermolt, a molt inhibiting hormone produced by the x organ is present which suppresses the y organ. Ecdysones have been measured in *Callinectes sapidus* during three stages of the molt (Faux *et al.*, 1969). The hormones were lowest in intermolt, higher during proecdysis, and highest just after ecdysis when the crab was soft. The MFO activity in crab green glands was inversely related to the levels of ecdysones, highest in intermolt, falling during proecdysis, and lowest just after the molt when the crab was soft (Figure 1). Immediately following ecdysis, MFO activity increased rapidly when the levels of ecdysones undergo an opposing decrease. An important ecdysone in crabs is crustecdysone, which is a 20-hydroxy derivative of a cholesterol-like presursor. Thus, steroid hydroxylase activity by the MFO system in the green gland could regulate levels of this hormone.

If mixed function oxygenases help to control molting hormone levels, then foreign organic compounds, such as polycyclic aromatic hydrocarbons, could compete with molting hormones at the active site of MFO and thus alter the rate at which crabs pass through early molts. This competition may explain why juvenile crabs are much more sensitive to pollutants than aduts (Armstrong *et al.*, 1976; Karinen and Rice, 1974; Nimmo *et al.*, 1971).

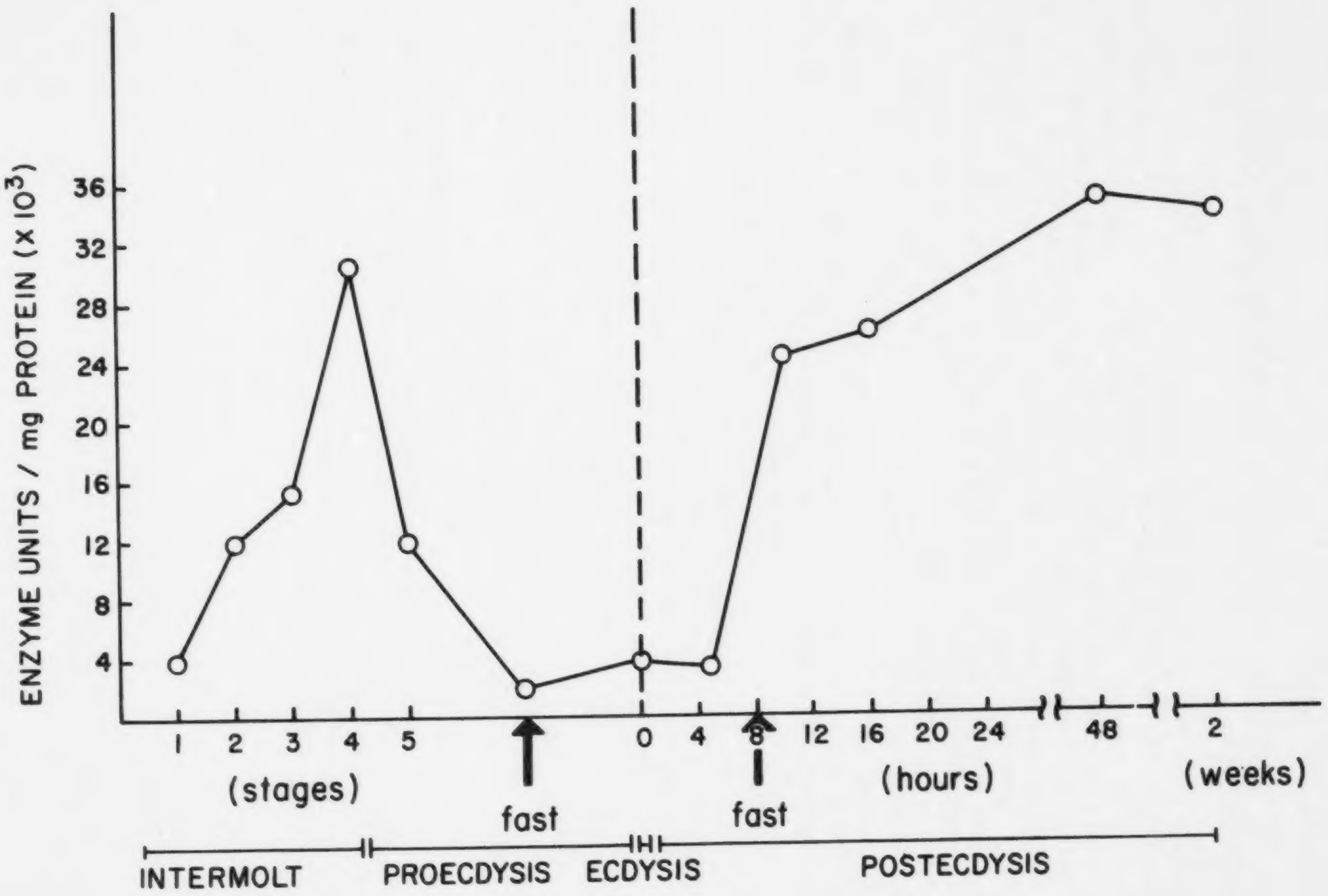

Figure 3. Flucation of mixed function oxygenase activity during molting in the green gland of female blue crabs. Intermolt stages were arbitrarily judged by appearance of molting rings followed by a fast beginning 3 to 7 days prior to ecdysis. Postecdysis was measured from the moment when newly molted crab was free of old carapace (from Singer and Lee, 1977).

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TECHNIQUES FOR THE WATERBORNE ADMINISTRATION OF BENZO(A)PYRENE TO AQUATIC TEST ORGANISMS

by

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ABSTRACT

Benzo(a)pyrene (BaP) has been used in many experiments to study its uptake by and effects on aquatic organisms. BaP has been reported to be sparingly soluble in water (4 to 12 μg/ℓ). However, even these low levels do not persist because of adsorption to surfaces or to particulate matter in test systems. Addition of BaP to water with small amounts of solubilizing carriers (i.e. ethanol, methanol, benzene) does not appreciably increase the amount in solution because BaP tends to precipitate after contact with water.

Two techniques reported in this paper describe how to make BaP more available to aquatic test organisms and increase its water solubility. A technique has been developed in which the compound is thinly coated onto sand particles to provide a large surface area of BaP crystals in water contact. Although water concentrations are very low, benthic test organisms are exposed to large quantities of BaP while resting on or burrowing into the sand.

Another technique has been developed in which BaP is made water soluble by entrapping the compound in a water soluble carrier. Bovine serum albumin (BSA) is used as a physical carrier and concentrations of up to 200 ppb of BaP can be effectively dissolved in water without precipitating any of the BaP. Although the concentration of the BaP-BSA complex decreases with time due to breakdown and adsorption onto particulates or glass surfaces, this complex remains dissolved in water longer than crystalline BaP. BaP levels can be adequately maintained in the water by regular additions of the BaP-BSA complex.

INTRODUCTION

One of the more carcinogenic of the polycyclic aromatic hydrocarbons (PAH) is 3, 4 benzo(a)pyrene (BaP) which occurs ubiquitously in the environment (Andelman and Suess, 1970). BaP in the environment has been associated to a large extent with man-controlled activities, such as coal burning, open burning, or automobile exhausts, and to a lesser extent, with synthesis by lower organisms. An important characteristic of BaP is its extremely low solubility in water, ranging from 4 to 12 up to 53 parts per billion (ppb), depending on the conditions (Davis *et al*., 1942; Wolk and Schwab, 1968).

BaP solubility in water can be increased by incorporating it into detergent micelles or by introducing BaP into the water with fairly large quantities of water-soluble organic solvents such as ethanol, acetone, dioxane, or methanol (Suess, 1972a). The solubility of BaP in water can also be increased by adding other compounds such as lactic acid, amino acids, purine bases, and other PAHs (Bohon and Claussen, 1951; Andelman and Suess, 1970).

Other investigators have used more complex organic molecules and mixtures such as horse serum, albumin, or DNA to bind PAHs (Liquori *et al*., 1962; Sahyun, 1964; Bothorel and Dismazes, 1974; Ceas, 1974). Serum albumin's ability to complex small molecules is well-known and has been extensively studied by pharmacologists and toxicologists. Only in recent years has the technique been employed by those conducting bioassays (Sanborn and Malins, 1977).

Another important characteristic of BaP is its ability to adsorb on surfaces. BaP has been shown to concentrate onto activated carbon, calcite material, silica glass, and plastics (Wolk and Schwab, 1968; Andelman and Suess, 1971). The presence of minerals or suspended or settled particulates in the water will greatly influence the distribution and solubility of BaP.

The importance of BaP in the aqueous environment has led to a number of laboratory experiments designed to study bioaccumulation, toxicity, or changes in metabolic patterns in a variety of aquatic organisms (i.e., Clark and Diamond, 1971; Lee *et al*., 1972; Couch and Winstead, 1979). However, many of the bioassays have shown variable results because of the absence of standard exposure and analytical methods and also because of difficulties involving solubilization, quantitation, and degradation of BaP in water. The amount of BaP that can be added to pure or saltwater is also a function of many factors such as temperature, salinity, and amount and duration of mixing. Once in the water, BaP is subjected to photodegradation or adsorption to particulate matter and this will considerably alter the concentrations and distribution (Andelman and Suess, 1970).

Researchers have used organic carriers such as benzene, methanol, acetone, or ethanol to help solubilize BaP (Suess, 1972a; 1972b).

However, addition of BaP to water with small amounts of solubilizing carrier does not necessarily increase the amount in solution because BaP tends to precipitate out on water contact. Fairly large proportions or organic carriers, such as were used in the study by Suess (1972a), are not feasible for bioassays since even small amounts of solvent could affect the metabolic patterns of the organism under study.

Our investigation was carried out to explore two possibilities of making BaP more available to aquatic test organisms without organic solvents.

MATERIALS AND METHOD

Coating Benzo(a)pyrene on Sand

BaP* was coated onto sand particles (8 to 10 mesh) by the following procedure. Silica sand (1000 g) was first washed with soap and water to remove dirt and debris, and then rinsed three times with water and acetone. Washed sand was spread evenly on a flat tray and dried in an oven (110° C) overnight. BaP was prepared by weighing approximately 500 mg and dissolving it in 150-200 mℓ of methylene chloride in a 1-ℓ Erlenmeyer flask. The dried sand (cooled to room temperature) was then added to the methylene chloride/BaP solution and stirred. The flask was placed in a shallow water, and the methylene chloride was evaporated to dryness by a nitrogen stream. Subsequently the flask was stoppered and placed in the dark. BaP-coated sand was placed either in the bottom of the test aquarium or in a piece of glass tubing incorporated in the water delivery system in a high flow rate to maximize the opportunity for BaP to dissolve in the water.

Analysis of BaP in Water

A measured volume of saltwater (usually 5 to 50 mℓ) was taken from the aquarium with a volumetric pipette and filtered through a methylene chloride washed, 0.45-μ Gelman filter. The filtered water was then placed in a 250-mℓ separatory funnel and extracted three times with one-half the sample volume of methylene chloride. The organic solvent portions were then combined, made up to a known volume in a volumetric flask (50 to 100 mℓ), adequately mixed, and a portion of this solution was analyzed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The excitation wave length was at 365 nm (10 nm silt), and the emission at 405 nm was used to quantitate the amount of BaP in methylene chloride. Appropriate dilutions of pure BaP dissolved in methylene chloride were used as standards and analyzed under the same conditions.

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*Aldrich Chemical Company, Milwaukee, WI

BaP Binding to Albumin

The procedure of Bothorel and Demazes (1974) was modified and used in our studies to bind BaP to Bovine Serum Albumin (BSA): 2-chloroethanol (75 mℓ) was slowly added to 50 mℓ of an aqueous solution containing 250 mg BSA and allowed to equilibrate for 10 min. A solution of BaP in toluene (9.7 mg/2.5 mℓ) spiked with 10 μCi of ^{14}C BaP was then added dropwise to the BSA-chloroethanol solution with vigorous stirring and this mixture was immersed in a liquid nitrogen for 3-4 min.

The frozen solution (about 127 mℓ) was thawed and then placed on a rotary evaporator to remove chloroethanol and water. The volume of this solution was reduced to 25 mℓ and the contents were placed on a gel filtration column (4.5 cm x 75 cm) packed with sephadex G-25, which was thoroughly washed with distilled water prior to use.

The albumin-BaP complex was eluted at the void column separating it from the unbound BaP. The BaP-BSA complex eluted from the gel filtration column was lyophilized, reconstituted in 25 mℓ water, and relyophilized three times. According to Bothorel and Desmazes (1974), repeated freezing and drying restores the protein conformation. The BaP-BSA complex was stored in the dark in a dry form before use in the experiments. BaP was absorbed onto BSA, a peptide chain and whale myoglobin without the aid of 2-chloroethanol treatment by a simple absorption technique as described in the BaP-BSA complex for the solubility study below.

Solubility Studies

The discrepancy in the literature between the various investigator claims on seawater solubility of BaP necessitated a carefully controlled and optimized solubility test. The first experiment was performed with "Marine Environment,"[*] an artificial seawater previously adjusted to a salinity of 1.022 and filtered through a 0.45-μ filter and carbon. The water contained no organics. It was then purged with ultra-pure nitrogen for 15 min prior to addition of 20 μg of BaP. The flask was protected from light and slowly stirred for 65 hr. The seawater was filtered through a 0.45 μ filter and the flask allowed to dry. The BaP concentration in the water was measured as previously discussed. Deposits on the flask wall were extracted into methylene chloride and measured. The amount adhering to the stirring bar was also measured, since Teflon has a high affinity for BaP. Care was taken to achieve the best solubility of BaP under these conditions.

The next set of experiments was done in beakers to test BaP solubility under three different conditions. In the first experiment, 20 μg of BaP in 100 μℓ ethanol was rapidly stirred into a liter of artificial seawater and allowed to stir slowly for periodic measurements during seven days. Additional injections of 20 μℓ each were added during the seven-day period to study photodegradation.

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[*] Import Associates, Inc., San Francisco, CA 94116

The purpose of the second experiment was to determine the absorption capabilities and resultant solubility of BaP bound to BSA without the aid of 2-chloroethanol. The BaP-albumin complex was made by adding 252 μg BaP dissolved in 100 μℓ ethanol to a solution of 120 mg BSA in 30 mℓ water. This stock solution was allowed to stir for 30 min and then added to 1ℓ of artificial seawater and stirred for seven days. The concentration of BaP was measured in filtered water samples on the first, fourth, sixth, and seventh days. The third experiment used sand coated by BaP as previously described (40 g BaP-coated sand was placed in a 1-ℓ beaker of artificial seawater and allowed to stir at room temperature for seven days). Filtered samples were measured periodically during the seven days.

The solubility of the BaP-BSA complex in seawater was also determined by the method of Bothorel and Demazes (1974). A known concentration of the complex was placed in an artificial seawater aquarium and circulated 9 days by a submersible. The aquarium contained no fish. A filtered and unfiltered sample was assayed periodically for the 9-day period as previously described.

UPTAKE OF BaP-BSA COMPLEX BY TEST FISH

Lyophilized BaP-BSA Uptake--Two adult English sole were forced-fed capsules containing 6.0 mg dry lyophilized BaP-BSA complex labeled with ^{14}C-benzo(a)pyrene. This work was performed before the water-borne study in order to ascertain whether the fish would assimilate the BaP after proteolytic digestion of the BaP-BSA complex. Fish were sacrificed after 24 hr and selected tissue was solubilized with protosol.* Aquasol* was then added to the solubilized tissue and the amount of radioactivity was measured in a liquid scintillation counter.

Water-borne Uptake of BaP-BSA Complex from Seawater--Two 24-ℓ aquaria containing artificial seawater were used for this experiment. The BaP-BSA complex was added to one tank to give an initial concentration of 1.7 μg/ℓ of BaP (2.4 mg/ℓ BSA; this concentration of BaP also contained 1.07 x 10^{-3} μCi ^{14}C BaP/μg unlabeled BaP). The second tank contained only BSA which had undergone a similar 2-chloroethanol treatment minus the BaP. A submerged circulating pump was placed in each tank and the system was allowed to equilibrate overnight. Aliquots (50 mℓ) of water were then removed, extracted with methylene chloride, and analyzed for BaP.

Three adult English sole were placed in the control and the experimental tanks. After three days, the fish were sacrificed and the liver, kidney, brain, and portions of the skin and muscle tissues were taken, solubilized with protosol, and the amount of radioactivity present in these organs was measured by liquid scintillation counting.

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*New England Nuclear

RESULTS

Solubility Studies

The experiments with BaP-coated sand showed that very low levels (about 1 ppb) were present in the water either in a sand column or in sand placed at the bottom of the aquarium. In both cases, however, small crystalline particles of BaP, which had sheared off from the sand, were observed suspended in the water. These suspended particles were filtered off before any concentration measurements were conducted.

Although levels of BaP in the water were very low, test fish resting on or burrowing into the sand accumulated substantial amounts of BaP on their integumental surfaces. Concentrations of BaP found adsorbed onto integumental surfaces ranged from 14 ng to 4 μg. A discussion of the adsorption of BaP on the skin and the uptake of BaP by flatfish tissue is presented in another paper in this symposium (Landolt et al., 1978).

In the solubility study with crystalline material, BaP was dissolved in ethanol and added to saltwater to obtain an initial concentration of 20 ppb. This level of BaP decreased to less than 1 ppb after 24 hr. Even with repeated additions of 20 ppb BaP to the water, there was no increase in the overall concentration, and levels in the water remained below 1 ppb (Figure 1).

In the BaP-albumin solubility study, the complex dissolved completely in saltwater, and an initial concentration of 16 ppb was obtained. Analysis of the BaP concentration in the water after 2, 4, 6, and 7 days showed that the concentration was 16, 10, 6, and 5 ppb, respectively (Figure 1).

The complexing ability of BaP to unmodified BSA and 2-chloroethanol treated BSA is shown in Table 1 and illustrates the difference between absorption of BaP on BSA to that of a smaller protein such as whale myoglobin and oxidized B-chain of insulin.

TABLE 1. COMPARISON OF THE COMPLEXING CAPACITY OF ALBUMIN FOR BaP BY SIMPLE ABSORPTION AND BY 2-CHLOROETHANOL TREATMENT

Substance	Treatment	Complexing capacity mg BaP/mg protein
Albumin + BaP	2-chloroethanol	3×10^{-2}
Albumin + BaP	Absorption	4×10^{-5}
Insulin + BaP	Absorption	3×10^{-5}
Myoglobin + BaP	Absorption	8.2×10^{-6}

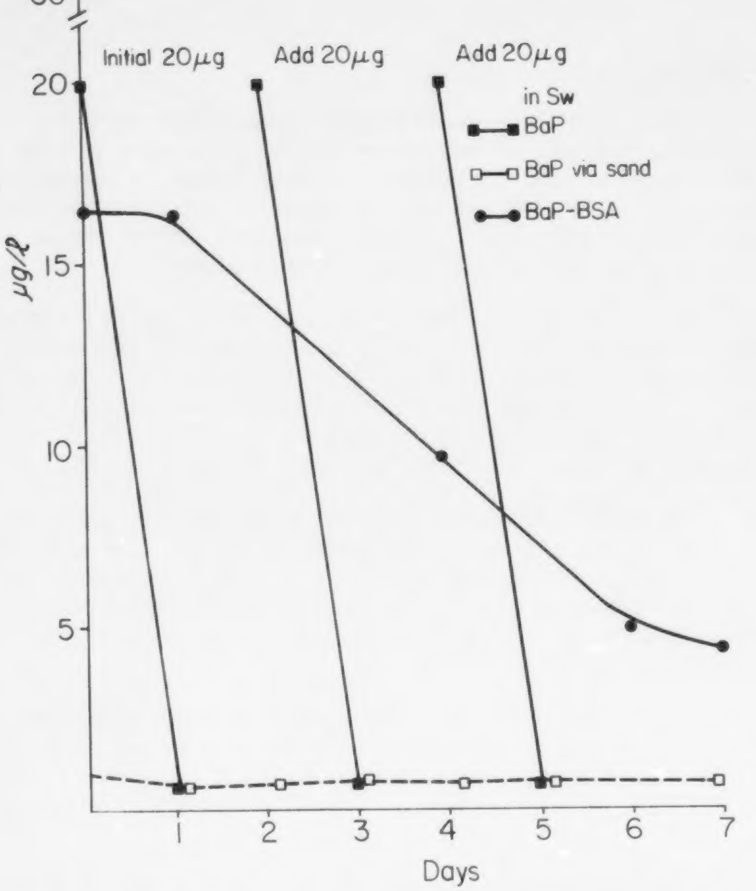

Figure 1. Solubility comparison of BaP in seawater via three methods of introduction.

The stability of the BaP-BSA complex in saltwater (Figure 2) followed a decay curve similar to that obtained by simple absorption and stirred in a flask (Figure 1). However, the initial concentrations are considerably different. The rate of decay of the solubilized BaP-BSA complex is much slower that that of the unfiltered complex. This can be accounted for in the circulating aquarium system by the action of the sand filter on the unbound BaP or crystalline BaP.

Figure 3 diagrammatically summarizes the distribution of BaP injected into a marine system without the aid of a solubilizer. The 10% soluble fraction is a liberal figure based upon the solution of BaP in saltwater under optimum conditions. This figure illustrates what occurs when BaP dissolved in an organic solvent such as ethanol is injected into saltwater.

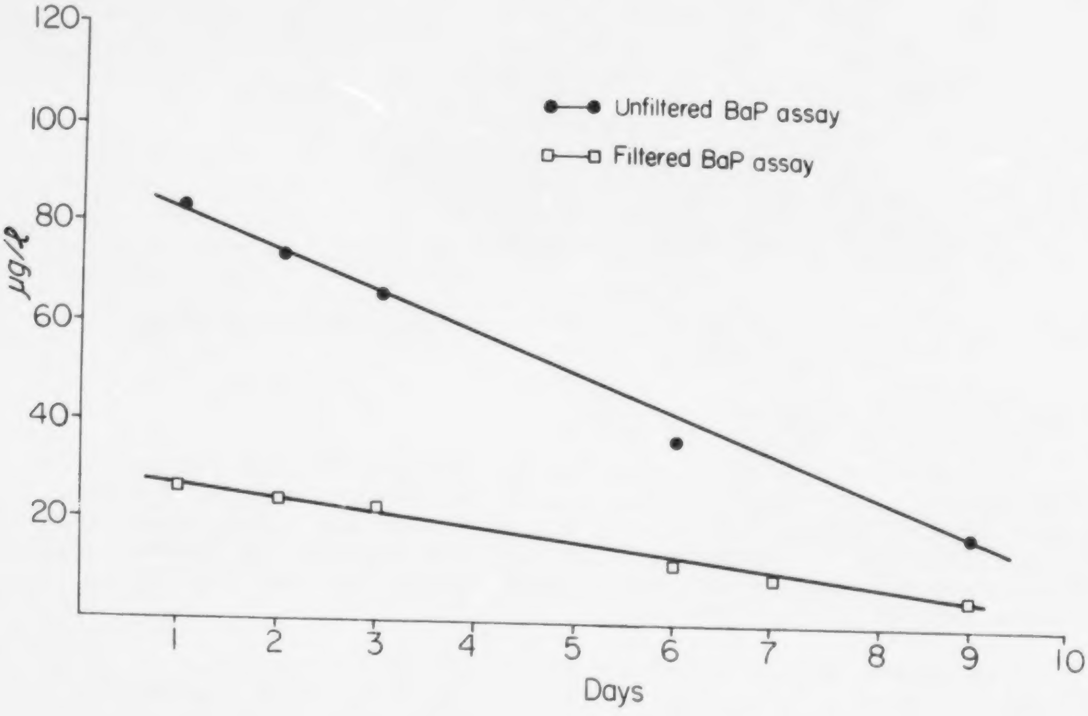

Figure 2. Stability of BaP-BSA complex in a static aquarium system.

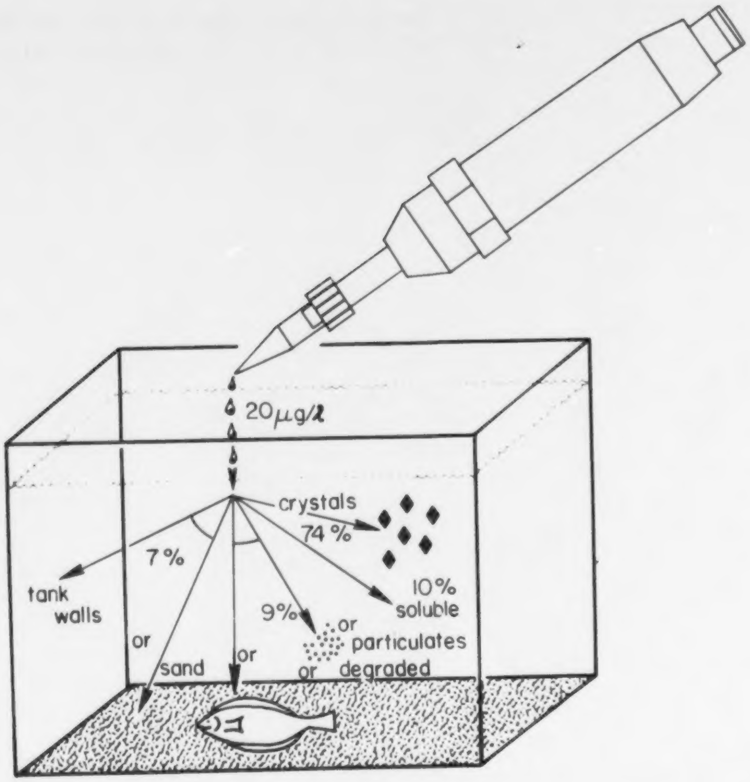

Figure 3. Schematic distribution of BaP in an aquarium system using an alcoholic concentrate of BaP.

Uptake of BaP-BSA Complex by Flatfish

The results of the forced feedings with encapsulated BaP-BSA were quite dramatic. Within 24 hr after feeding the BaP was released from the albumin binding and was found widely distributed in the various organs and tissue. This clearly demonstrated that the fish digested the albumin, that the released BaP could be absorbed by body tissues, and that the complex could be used in uptake experiments.

A waterborne BaP-BSA experiment had an initial concentration of 1.7 µg/ℓ (assayed as BaP) added to the 98-ℓ aquarium. Table 2 shows the results of the uptake of ^{14}C-labeled BaP in three fish. High concentrations of BaP or its metabolites were found in the kidneys, liver, and skin. Histopathological examination of the tissues from the fish were also undertaken.

Tissues from the control fish were essentially normal, but tissues from the experimental fish revealed a number of lesions. Liver sections contained large numbers of hepatocytes with clear cytoplasmic vacuoles.

These vacuoles were anatomically distinct from those associated with stored lipid or glycogen and were most frequently clustered in peripancreatic zones. Vascular sinusoids were indistinct, and there were focal, although much smaller, areas of necrosis, as in control specimens. The livers of these fish were also heavily infested by a sporozoan parasite, *Myxidium* sp. The kidney tissue contained multiple foci of pyknosis and necrosis within hematopoietic tissue. The renal tubules and glomeruli were as expected. The gills contained a limited number of hyperplastic lamellae in which there was epithelial proliferation at the distal tips. Several aneurysms and encysted dinoflagellate parasites belonging to the genus *Oodinium* were also present. Focal dermatitis was of limited extent, and the heart contained an encysted helminth. The other organs were unremarkable.

TABLE 2. UPTAKE OF ^{14}C-LABELED BaP-BSA COMPLEX (1.7 μg/ℓ OF ASSAY BaP) BY ENGLISH SOLE FOLLOWING 7-DAY WATER-BORNE EXPOSURE

Fish 1 Tissue	Wet wt. used	CPM	μCi in sample	μg BaP per gm of wet wt.
Liver	0.1008	369	2.3×10^{-4}	2.16
Brain	0.0497	90	4.3×10^{-5}	0 81
Muscle	0.1113	91	5.3×10^{-5}	0.44
Skin	0.0923	324	2.2×10^{-4}	2.24
Kidney	0.1047	928	5.8×10^{-4}	5.16
Fish 2 Tissue				
Liver	0.1025	898	5.6×10^{-4}	5.10
Brain	0.1018	103	6.2×10^{-5}	0.57
Muscle	0.1025	116	6.7×10^{-5}	0.61
Skin	0.1119	484	3.3×10^{-4}	2.75
Kidney	0.1012	1526	9.5×10^{-4}	8.84
Fish 3 Tissue				
Liver	0.1078	969	6.1×10^{-4}	5.24
Brain	0.1034	91	5.5×10^{-5}	0.50
Muscle	0.0974	80	4.6×10^{-5}	0.44
Skin	0.0943	425	2.9×10^{-4}	2.88
Kidney	0.1021	790	4.5×10^{-4}	4.53

DISCUSSION

Solubility

Our results show that even with a large quantity of BaP coated on sand, the concentration of water-soluble BaP remained very low. Although the solubility of BaP in water has been reported to be from 4 to 12 ppb (Davis et al., 1942), studies in our laboratory have shown that 16 to 20 μg/ℓ of crystalline BaP can be dissolved in seawater with vigorous shaking. However, the concentration in the water decreased to about 1 ppb after several hours.

Even when BaP was added with an organic solvent such as ethanol, levels of BaP in solution decreased to near zero after 24 hr. Repeated additions of the same concentrations of BaP in ethanol into the water showed no increase in BaP concentrations. Andelman and Suess (1971) reported a loss of BaP from the water to boro silicate glass surfaces at a rate of about 8 ng per cm^2 per 3-hr period. For Teflon and plexiglass, losses were reported to be 38 and 50 ng per cm^2/3 hr, respectively.

These results show that it is very difficult to maintain any concentration of BaP in solution for any length of time, particularly in systems with glass, Teflon, or PVC surfaces.

On the other hand, the binding of BaP to large molecules, such as BSA (Bothorel and Desmazes, 1974) or DNA (Liquori et al., 1962), greatly increases stability and the solubility of BaP in water. Although the concentration of the BSA-BaP complex in our test system decreased from 16 ppb to 5 ppb over a 7-day period (70% decrease), this latter concentration is still five times higher than the concentration that can be obtained by dissolving crystalline BaP in water.

This experiment also shows clearly that the BSA-BaP complex will persist for a fairly long time as compared to the addition to water of crystalline BaP.

The decrease in BaP concentration, due either to adsorption to surfaces or to bacterial degradation of the protein, could be easily increased to original levels by the dropwise addition of the BaP-BSA complex dissolved in water. Concentrations of the BaP-BSA complex in water can still be easily measured by fluorometric analysis since the BaP adsorption onto proteins does not significantly alter the fluorescence spectrum of BaP between 250 and 450 nm.

The technique of utilizing a carrier for a compound, such as BaP, and maintaining the concentration of dissolved BaP in aqueous systems seems to be an excellent method for conducting water-borne experiments with low levels of hydrophobic compounds.

Uptake Studies

Bap Coated on Sand--Although very little BaP dissolved in the saltwater from the coated sand, this coating technique did provide a fairly large source of crystalline BaP that can be taken up by the test animals, especially benthic organisms.

Our results (Table 3) clearly indicate that test organisms accumulated some of the BaP adsorbed onto the sand or sediment on their integumental surfaces. Presumably, any small particles of crystalline BaP in the sand will adhere to the mucous layer on the skin surface as the flatfish burrows or buries itself in the sand. The amounts of BaP found in the methylene chloride wastes vary from 15 ng up to 4.4 μg per fish. This large variability in BaP level suggests that there is continual uptake and release of the compound from the mucous surface.

TABLE 3. CONCENTRATIONS OF BaP FOUND IN METHYLENE CHLORIDE WASHES OF INTEGUMENTAL SURFACES OF ENGLISH SOLE FOLLOWING 30-DAY EXPOSURE TO BaP-COATED SAND

Fish Number	Total Weight of Fish Analyzed(g)	Total BaP Found in Methylene Chloride Washes (ng)[1]
6 (control)	20.1	N.D.[2]
7 (exptl)	10.2	285
8 (exptl)	5.6	414
9 (exptl)	31.3	230
10 (exptl)	46.3	395
11 (exptl)	6.9	993
12 (exptl)	11.2	4,450
13 (exptl)	4.9	15
14 (exptl)	69.6	1,695

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[1] Determined by gas chromatography analysis (Landolt *et al*., 1978).
[2] Not detectable

The results from the analysis of the tissues of flatfish exposed to BaP-coated sand indicate that there was BaP uptake by the organisms and measurable amounts of the parent compound were present in the whole body tissues after 30 days (Landolt *et al*., 1978). There has been no study to show whether BaP or other hydrophobic compounds can be transported from the external surfaces through the scale and skin and be either stored or metabolized by marine flatfish. Since marine fish must drink seawater to osmoregulate, particles of BaP could be introduced into the gastrointestinal tract by this process and be absorbed. Another path of uptake of BaP is through the gills. In a study of BaP uptake and metabolism by marine fish, Lee *et al*. (1972) found high levels of BaP accumulation in gill tissues. This finding led to the postulation that the path of BaP uptake would be through the gills followed by accumulation of the hydrocarbon and its metabolites by the organs and tissues.

BSA-BaP Complex--These studies were conducted to determine if BaP bound to BSA could be absorbed and metabolized. Other studies (Ceas, 1974; Sanborn and Malins, 1977) have shown that certain hydrophobic compounds bound to BSA or horse albumin can be absorbed and metabolized. Sanborn and Malins (1971) have shown that larval spot shrimp (*Pandalus platyceros*) and larval Dungeness crabs (*Cancer magister*) absorbed and metabolized ^{14}C naphthalene bound to BSA. Ceas (1974) found that a water-soluble horse albumin-BaP complex adversely affected the development of sea urchin eggs. Our data clearly show that radioactivity from the BaP was distributed throughout selected tissues and organs, indicating that the test organisms were able to absorb BSA-bound BaP.

The higher concentrations of radioactivity found in livers and kidneys indicate that these organs are the more active of the tissues in metabolizing and storing BaP. Unpublished results form our laboratory (Landolt *et al*., 1978) show that livers of English sole exposed to BaP via intraperitoneal injection rapidly accumulated BaP during the first day, but no further increase in radioactivity was noted during the next seven days. This is similar to the results obtained by Lee *et al*. (1972) who also noted a steady-state level in livers of *Gillichthys mirabilis* exposed to ^{3}H-labeled BaP.

These studies show that BaP can be administered to test organisms by coating the sediment or by complexing the compound to a large water-soluble molecule such as BSA. The coated sediment technique is an excellent method for benthic test organisms since there is intimate contact between the organism and the compound. On the other hand, the BaP-BSA complex method allows increased water solubility of the test compound and provides a higher exposure concentration to epibenthic and pelagic organisms.

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ACTIVATION AND UPTAKE OF POLYNUCLEAR AROMATIC HYDROCARBONS BY THE MARINE CILIATE, *PARAURONEMA ACUTUM*

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ABSTRACT

The marine ciliate, *Parauronema acutum*, can convert 2-aminofluorene and 2-acetylaminofluorene to compounds with mutagenic activity in the Ames/*Salmonella* test. The ciliate, however, does not activate benzo(a)pyrene or benzanthracene or destroy the mutagenic properties of nitrosoguanidine. Homogenates when substituted for a liver microsome fraction (S-9) in the *Salmonella*/microsome test activate 2-aminofluorene and 2-acetylaminofluorene to mutagens. Benzo(a)pyrene (BaP) and benzanthracene are not activated nor is nitrosoguanidine inactivated. Phenobarbitol does not induce or increase the amount of activating activity. The activating activity shows no requirement for the NADPH regenerating system required by liver microsomes and hence may not be due to a typical mixed function oxidase. Upon differential sedimentation of cell homogenates, the majority of the activity sediments along with a small particulate fraction that has sedimentation properties of microsomes parallel to those of higher eukaryotes. BaP though not metabolized is accumulated by cultures of *P. acutum* at a linear rate but not released after removal of BaP from the incubation medium to a great degree (10%). Hence, this ciliate can convert certain polynuclear aromatic hydrocarbons to mutagens and accumulate others, such as BaP, which it cannot metabolize. The importance of these facts is dependent on the population of this ciliate in marine environments and the likelihood of contact between ciliate and environmental contaminant.

INTRODUCTION

The importance of polynuclear aromatic hydrocarbons as contaminants of the marine environment has become more evident in recent years. The effects of marine organisms on these compounds has only recently become an

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area of investigation. The abilities of higher eukaryotes to alter and accumulate compounds, such as benzo(a)pyrene and aminofluorene, have been studied in some detail. No study on the alteration of compounds or their uptake by marine single cell eukaryotes has been udertaken. Their importance, possibly because of their high population in marine environments and their primary position in marine food webs, cannot be underestimated. We have been able in this study to investigate the uptake and alteration of polynuclear aromatic hydrocarbons which may be at significant levels in the marine environment.

MATERIAL AND METHODS

Organism and growth conditions--The marine ciliate, *Parauronema acutum*, was obtained from Dr. A.T. Soldo, Veterans Administration Hospital, Miami, FL. The ciliate was grown in the dark at 24° C in seawater M medium (Soldo and Merlin, 1972). Cells (72-hr-old) for experimentation were collected by centrifugation at room temperature (1000 rpm for 4 min in the HNS-II centrifuge, Daman/IEC) and either washed (2 times) and resuspended in 0.25 M sucrose (cell fractionation studies).

Mutagenesis--The mutagenic activity of compounds [benzo(a)pyrene, 2-aminofluorene, 2-acetylaminofluorene, nitrosoguanidine, benzanthracene, and the solvent in which the compounds were dissolved--dimethylsulfoxide] and the ability of growing cells and cell homogenates to convert these compounds to mutagens or eliminate their mutagenic properties was as described by Ames *et al*. (1975). Histidine revertants as a measure of mutagenicity were enumerated using *Salmonella typhimurium* tester strains TA 98, TA 100, TA 1337, TA 1535, TA 1537, and TA 1538 obtained from Dr. Bruce N. Ames, Biochemistry Dept., University of California, Berkeley, CA. Strains Ta 100, TA 1535 are used to detect mutagens causing base-pair substitutions and TA 98, TA 1538, TA 1537 for the detection of various kinds of frameshift mutations (Ames *et al*., 1973a).

Conversion of compounds by growing cells--The compounds (listed above) were added to 48-hr cultures of *P. acutum* at a final concentration of 500 ng/mℓ. After an additional 24-hr growth, the cells were removed by centrifugation and the culture medium tested for mutagenic activity in the Ames test (Ames *et al*., 1973a). Control cultures had the tested compound added after 72-hr growth, just prior to removal of cells by centrifugation.

Conversions of compounds by cell homogenates and fractions--Homogenates and fractions obtained by differential centrifugation of homogenates were tested for their ability to convert compounds into mutagens by incorporation into *Salmonella*/microsome test of Ames (Ames *et al*., 1973a). The homogenates or fractions were substituted for the S-9 fraction used in the *Salmonella*/microsome test as a source of activating (microsomal) enzymes (Ames *et al*., 1975; Durston and Ames, 1974). The homogenates or fractions were incorporated into the top agar with the compound to be tested and the *Salmonella* tester strain (Ames *et al*., 1973a, 1975). Revertants were enumerated after 2 days incubation at 37° C.

Enzyme assays--Malate dehydrogenase activity was measured by the oxidation of NADH (No. O.D. 340 nm) with oxalacetate as a substrate (Lindmark and Muller, 1974). Acid phosphatase and β-N-acetylglucosaminidase were measured by release of p-nitrophenol (No. O.D. 410 nm) (Lindmark and Muller, 1974). Pyruvate kinase was measured by phenylhydrazone production from phosphoenol-pyruvate (Prichard and Scofield, 1968). NADPH-cytochrome c reductase was measured by reduction of cytochrome c (Masters *et al.*, 1967) Activation of 2-aminofluorene or 2-acetylaminofluorene was measured by enumerating the number of histidine revertants produced in tester strain TA 1538. The 2-aminofluorene (10 µg) or 2-acetylaminofluorene (10 µg) were incorporated with the enzyme sample into the top agar with the tester strain as described by Ames (1973a, b; and Durston and Ames, 1974). Results presented were obtained from experiments containing optimal concentrations of enzyme and test compound. Catalase was measured by disappearance of H_2O_2 (Muller, 1973). Fumarase is measured by No. O.D. 240 with malate as a substrate (Hill and Bradshaw, 1969).

Cell fractionation--Homogenates of *P. acutum* were prepared by five successive passages of cells suspended in cold 250 mM sucrose through a 20 µm average pore size stainless steel filter. Differential centrifugation of the homogenate in a refrigerated centrifuge (SS-34 rotor) (Dupont-Sorvall, Norwalk, CT) resulted in four fractions: 3 particle fractions sedimenting at 500 rpm for 4 min, 2500 rpm for 10 min, 19,000 rpm for 60 min, and a nonsedimentable fraction not sedimenting at 19,000 rpm. This sedimentation procedure resulted in the separation of particles sedimenting at 2500 rpm containing mitochondrial (malate dehydrogenase, fumarase) and peroxisomal (catalase) enzymes from those particles sedimenting at 19,000 rpm containing hydrolytic (acid phosphatase, β-N-acetyl glucosaminidase) and microsomal (NADPH-cytochrome C reductase) enzymese. All particle associated enzymes are separated from the non-sedimentable cytoplasm (pyruvate kinase).

Uptake and release of benzo(a)pyrene--The 72-hr cells were harvested by centrifugation and resuspended in half the original volume of artificial seawater. ^{14}C benzo(a)pyrene (0.04 µCi/mℓ) final concentration was added, and the sample shaken at 24° C at 200 rpm in a shaking water bath (Aquatherm, New Brunswick Scientific, New Brunswick, NJ) by centrifugation and washed twice in seawater. The pellet was resuspended in 1.2 mℓ of 0.1 NaOH containing 0.4% deoxycholate. A 100 µℓ sample was added to 10 mℓ Bray's solution and radioactivity determined by scintillation counting.

After 4 hr in the presence of ^{14}C benzo(a)pyrene, the remaining cells were washed free of benzo(a)pyrene (2 times) and resuspended in the original volume of seawater. Aliquots were taken at hourly intervals, and the remaining ^{14}C label in the cells was determined as described above.

Chemicals--Artificial seawater was obtained from Aquarium Systems, Eastlake, OH. Benzo(a)pyrene and 9,10-dimetyl-1,2-benzanthracene were obtained from Sigma Chemicals, St. Louis, MO. [1,10-^{14}C] Benzo(a)pyrene was obtained from Amersham, Arlington Heights, IL. N-methyl-N'-nitro-n-nitrosoguanidine, 2-aminofluorene, and 2-acetylaminofluorene were obtained from Aldrich Chemicals, Milwaukee, WI.

RESULTS

Conversions of compounds by growing cultures--As shown in Table 1, growing cultures of *P. acutum* can convert both 2-aminofluorene and 2-acetylaminofluorene to mutagenic compounds. Benzo(a)pyrene and benzanthracene are not converted nor is nitrosoguanidine converted into a non-mutagen. Dimethylsulfoxide (solvent for all compounds) was not converted into a mutagen. Tester strains TA 1537, 1538, and 98 are reverted, whereas TA 100, 1535 are not. This specific reversion suggests the occurrence of frameshift mutations for which these tester strains show a high sensitivity (Ames *et al*., 1973a; Durston and Ames, 1974).

Conversions of compounds by homogenates--If homogenates of *P. acutum* are incorporated with teest compound and tester strain TA 1538 into the *Salmonella*/microsome test, the results obtained are found in Table 2. 2-aminofluorene and 2-acetylaminofluorene are converted to mutagens. As with growing cells, benzo(a)pyrene and benzanthracene are not converted to mutagens nor is nitrosoguanidine converted into a non-mutagen. Tester strains TA 98, 1537 give similar results. TA 100 and 1535 exhibited no reversion above control. Homogenates prepared from cells grown in the presence of compounds (phenobarbitol, 1 mg/mℓ; 2-aminofluorene or 2-acetylaminofluorene, 500 ng/mℓ) known to induce and increase the cellular level of microsomal enzymes show no increase in activating activity and suggest the absence of induction (Ames *et al*., 1973a). The rate of activation on a per mg protein is 20 times that of Arochlor-induced S-9 fraction obtained from rat liver.

In order for full activity of the mammalian microsomal activating system of *P. acutum*, a NADP regeneration system is required. As shown on Table 3, the activating system of *P. acutum* does not have a requirement for the regeneration system. NADP, glucose-6-PO_4, Mg^{2+} are not required, suggesting the absence of a typical mixed function oxidase found in higher eukaryotes.

Differential sedimentation of homogenates--As shown in Table 4, aminofluorene activating activity shows the highest amount of activity in a small particle fraction, though 40% of the total activity is in the non-sedimentable cytoplasm. Further experimentation must be done to define the particle population with which the activating activity is associated, although there is a clear separation from the large particle fraction, sedimenting at a low speed, containing mitochondrial and peroxisomal enzymes.

Uptake and release of benzo(a)pyrene--Since growing cells and cell homogenates could not convert benzo(a)pyrene or benzanthracene into a mutagen, it became of interest to investigate the uptake and release of benzo(a)pyrene. *P. acutum* accumulates the label in benzo(a)pyrene at a

TABLE 1. CONVERSIONS OF COMPOUNDS BY GROWING CELLS OF *P. ACUTUM*[*]

	Histidine revertants per plate				
Compound	TA98	TA100	TA1535	TA1537	TA1538
2-aminofluorene	1250	199	32	1220	1095
control	49	152	36	21	24
2-acetylaminofluorene	1000	150	30	1100	1300
control	31	160	25	14	34
benzo(α)pyrene	24	140	23	18	22
control	20	160	18	19	31
benzanthracene	36	150	24	20	40
control	30	110	27	25	35
nitrosoguanidine	N.D.	1350	890	N.D.	N.D.
control	N.D.	1200	810	N.D.	N.D.
dimethylsulfoxide	18	140	29	16	30
control	19	162	36	15	28

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[*] Cells grown for 48 hr, compound added (500 ng/mℓ), cultures grown additional 24 hr, harvested, and supernatant solution tested for converted compound. Control contains an equivalent amount of compound added to culture after 72 hr growth in the absence of tested compound. N.D.=not determined.

TABLE 2. CONVERSION OF COMPOUNDS BY HOMOGENATES OF P. ACUTUM

Compound*	μg	Histidine revertants (TA1538)** per plate
2-aminofluorene	10	1240
control	10	25
2-acetylaminofluorene	10	1000
control	10	18
benzo(α)pyrene	50	39
control	50	29
benzanthracene	50	24
control	50	18
nitrosoguanidine	10	620
control	10	550

*Plates contain concentration of tester compounds as indicated and 0.4 mg of homogenate protein. Control has no homogenate added.

**TA 98, 1537 give similar results.

TABLE 3. REQUIREMENTS OF CONVERSION OF 2-AMINOFLUORENE BY HOMOGENATES OF P. ACUTUM

	Hisitidine revertants (TA 1538) per plate
Complete*	622
minus homogenate	16
minus 2-aminofluorene	18
minus NADP	628
minus glucose-6-PO_4	597
minus Mg^{2+}	630
minus NADP, Mg^{2+}, glucose-6-PO_4	620

*Contains: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-PO_4, 4 mM NADP, 100 mM Naphosphate (pH 7.4), 10 μg 2-aminofluorene, and 0.25 mg homogenate protein.

TABLE 4. DISTRIBUTION OF ENZYME ACTIVITIES AFTER DIFFERENTIAL SEDIMENTATION OF AN HOMOGENATE OF *P*. *ACUTUM*

	R.S.A.*						
Fraction	Catalase	Acid phosphate	BNAG	Fumarase	Malate denydrogenase	Pyruvate kinase	Aminofluorene activation
Nuclear	0.5	0.2	0.1	0.8	0.7	0.1	0.2
Large particle	2.1	1.0	0.4	3.2	3.6	0.3	1.0
Small particle	1.8	3.5	3.0	0.8	0.6	0.3	2.1
Non-sedimentable	1.6	0.3	0.8	0.7	0.5	2.1	1.0

*Percent enzyme/percent protein

linear rate for a period of 4 hr. After removing the benzopyrene from the environment, the cells lose 10% of the benzo(a)pyrene taken up in the first hour, but no more up to 48 hr. These results suggest that P. acutum can accumulate compounds, such as benzo(a)pyrene, which it cannot metabolize.

DISCUSSION

The marine ciliate, P. acutum, can convert amino polynuclear aromatic hydrocarbons, such as 2-aminofluorene and 2-acetylaminofluorene, to mutagens at a relatively high rate. Other compounds, such as benzo(a)pyrene and benzanthracene, are not altered. The activating activity does not require Mg^{2+}, glucose-6-PO_4 or NADP, suggesting that it is not due to a typical mixed function oxidase. The activity cannot be induced or increased by growing P. acutum in the presence of phenobarbitol, 2-aminofluorene, or 2-acetylaminofluorene.

The enzyme activity responsible for these processes may be due to a specific enzyme or, as suggested by the fact the P. acutum can use amino acids as its sole carbon source (Soldo and Merlin, 1972), may be due to enzymes that perform other physiological functions. The activity is associated with a small particle fraction which corresponds to the sedimentation properties of microsomes as isolated from other organisms. This activity can certainly add to the mutagen load in the marine environment if this ciliate occurs in a high population in an area in contact with aminofluorenes.

P. acutum can accumulate benzo(a)pyrene and possibly other compounds, but it cannot metabolize; this may be of importance in the marine environment proportional to the ciliate population and the amount of polynuclear aromatic hydrocabons.

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EFFECT OF POLYNUCLEAR AROMATIC HYDROCARBONS AND POLYHALOGENATED BIPHENYLS ON HEPATIC MIXED-FUNCTION OXIDASE ACTIVITY IN MARINE FISH

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ABSTRACT

The polynuclear aromatic hydrocarbons, 3-methylcholanthrene and dibenz(a,h)anthracene, induced hepatic microsomal benzo(a)-pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities in several marine fish, including sheepshead, *Archosargus probatocephalus*, little skate, *Raja erinacea*, southern flounder, *Paralichthyes lethostigma*, and stingray, *Dasyatis sabina*. The cytochrome P-450 content of hepatic microsomes from fish treated with dibenz(a,h)anthracene or 3-methylcholanthrene was usually not significantly higher than in control fish. 3-Methylcholanthrene and dibenz(a,h)anthracene had no effect on hepatic benzphetamine N-demethylase activity nor on epoxide hydrase or gluthathione S-transferase activity in these fish. The dose-response and time course of benzo(a)pyrene hydroxylase induction was studied in winter and in summer in sheepshead; the rate of induction was faster in summer. Commercial mixtures of polychlorinated biphenyls and polybrominated biphenyls also induced benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities and in most experiments caused a statistically significant increase in cytochrome P-450 content of hepatic microsomes. High individual variation between fish made it difficult to demonstrate significant differences with small increases or decreases in enzyme activities (2-fold less than control). These studies suggest that the control of cytochrome P-448-dependent mixed-function oxidation in marine fish is similar to that in mammals. However, the time course of induction is slower in fish, especially those acclimated to cold water.

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INTRODUCTION

As a result of oil spills and industrial pollution, marine species in coastal and estuarine environments are frequently exposed to polycyclic aromatic hydrocarbons (PAHs) and other chemicals, including polychlorinated biphenyls (PCBs) and even to polybrominated biphenyls (PBBs) in some areas. These chemicals induce their own metabolism in mammalian species (Conney, 1967; Dent et al., 1976; Litterst et al., 1972) and many are carcinogenic or mutagenic to mammals (Heidelberger, 1975). The effects of PAHs, PCBs, and PBBs on some hepatic xenobiotic-metabolizing enzymes in the rat are summarized in Table 1. In this paper we describe the effect of known doses of selected PAHs, and mixtures of PCBs or PBBs on hepatic xenobiotic-metabolizing enzymes in marine fish common to the Atlantic Ocean near Maine (studies carried out at the Mount Desert Island Biological Laboratory, Salsbury Cove, ME) or Northeast Florida (studies carried out at the C. V. Whitney Laboratory for Experimental Marine Biology and Medicine of the University of Florida, St. Augustine, FL). In addition we report the effects of a pure PCB isomer, 3,3'4,4',5,5'-hexachlorobiphenyl (3,3',4,4',5,5'-HCB), which is known to cause cytochrome P-448-dependent induction in mammals (Goldstein et al., 1977) and of a pure PBB isomer, 2,2',4,4'5,5'-hexabromobiphenyl (2,2',4,4',5,5'-HBB), which causes cytochrome P-450-dependent induction in rats (Moore et al., 1978).

MATERIALS AND METHODS

Animals--At both laboratories fish were caught locally and maintained in flowing seawater for the duration of each experiment. Species studied in Maine were the little skate, *Raja erinacea*, and the winter flounder, *Pseudopleuronectes americanus*. Species studied in Florida were the Atlantic stingray, *Dasyatis sabina*, the sheepshead, *Archosargus probatocephalus*, and the southern flounder, *Paralichthyes lethostigma*. In most experiments, five fish were injected intraperitoneally (i.p.) with a suspension of solution of the chemical under investigation and three fish were injected with an equivalent volume of the vehicle. In a few experiments the chemical was administered orally or by intramuscular (i.m.) injection. The day on which the compound was administered was designated "Day 1" of the experiment and time (days) to sacrifice varied with the compound, dosage, and time of year, as indicated in Results. All Florida fish were fed regularly throughout the experiments. At the designated time after dosage, animals were sacrificed and livers removed promptly and placed in ice-cold 1.15% KCl. Washed microsomes and cytosol fractions were prepared as previously described (James et al., 1979; Pohl et al., 1974).

Chemicals--3-Methylcholanthrene (3-MC), 7,12-dimethylbenzanthracene (DMBA), 1,2,3,4-dibenzanthracene (DBA), and hexadecane were obtained from Sigma Chemical Company. Aroclor 1254, Firemaster FF1, 1,3,3'4,4'5,5'-HCB, and 2,2'4,4'5,5'-HBB were generously supplied by Dr. J. McKinney, Laboratory of Chemistry, National Institute of Environmental Health Sciences.

TABLE 1. EFFECT OF CHEMICAL PRETREATMENT ON HEPATIC XENOBIOTIC METABOLISM IN THE RAT

CHEMICAL	MIXED-FUNCTION OXIDATION	EPOXIDE METABOLISM
Polynuclear Aromatic Hydrocarbons	Induce cytochrome P-448-dependent but not cytochrome P-450-dependent oxidations (Conney, 1967; Ryan et al., 1977)	Induce epoxide hydrase and GSH S-transferase activities (Oesch et al., 1973; Mukhtar and Bresnick; 1976; Bresnick et al., 1977; Hales and Neims, 1977)
Polychlorinated and Polybrominated Biphenyls	Induce cytochrome P-448 and cytochrome P-450 dependent oxidations (Litterst et al., 1972; Dent et al., 1976, Ryan et al., 1977)	PCBs and PBBs induce epoxide hydrase (Dent et al., 1976) and GSH S-transferase activity
3,3',4,4'-Tetrachlorobiphenyl 3,3',4,4',5,5'-Hexachlorobiphenyl	Induce cytochrome P-448 dependent oxidations (Goldstein et al., 1977)	Induce GSH S-transferase activity (Kohli et al.,1978)
2,2',4,4'-Tetrachlorobiphenyl 2,2',4,4',5,5'-Hexachlorobiphenyl 2,2',4,4',6,6'-Hexachlorobiphenyl	Induce cytochrome P-450 dependent oxidations (Goldstein et al., 1977; Moore et al., 1978)	Induce eposide hydrase and GSH S-transferase activities (Kohli et al., 1978)

Assay--Mixed-function oxidase activities measured in hepatic microsomes were benzo(a)pyrene hydroxylase (AHH), benzphetamine N-demethylase (BND), 7-ethoxycoumarin O-deethylase (7-EC), and 7-ethoxyresourfin O-deethylase (ERF) (James *et al*., 1979; Pohl *et al*., 1974). Cytochrome P-450 content and NADPH-cytochrome *c* reductase activity were quantitated as described previously (James *et al*., 1979). Epoxide-metabolizing enzyme activities measured were microsomal epoxide hydrase (EH), and cytosol fraction glutathione S-transferase (GSH-T); these activities were assayed as described by James and coworkers (1979). Epoxide substrates used were styrene 7,8-oxide, octene 1,2-oxide, and benzo(a)pyrene 4,5-oxide, although in most experiments styrene oxide was the substrate. In all cases, assay conditions used were those that gave maximum *in vitro* activity in the species under investigation.

RESULTS

Hydrocarbons

Effect on microsomal mixed-function oxidation--Administration of 3-MC to sheepshead, flounder, stingray, and skate resulted in induction of hepatic microsomal AHH and 7-EC activities. Table 2 shows results from four experiments with 3-MC. In other experiments with sheepshead and little skate, ERF activities were also elevated by 3-MC or DBA treatment. Mean cytochrome P-450 content of hepatic microsomes from 3-MC-treated fish was usually somewhat higher than control values, but the difference was seldom statistically significant due to wide variation between individual fish. Hepatic microsomes from only one species, the little skate, had elevated BND activities after 3-MC treatment. In other species, there was no difference in BND activities between treated and control fish. Treatment of sheepshead with DMBA (2 x 10 mg/kg, i.p. on Days 1 and 3) caused induction of AHH and 7-EC activities by Day 7, but did not affect cytochrome P-450 content or BND activity. Similarly, little skates treated with DBA (10 mg/kg, i.p. on Day 1) had induced AHH and 7-EC activities by Day 13, but cytochrome P-450 content was unaffected. However, cytochrome P-448, as well as cytochrome P-450, were isolated from hepatic microsomes of DBA-treated little skates (Elmamlouk *et al*., 1977).

AHH activity in hepatic microsomes from control fish was stimulated by *in vitro* addition of 7,8-benzoflavone (10^{-4} M), whereas AHH activity in hepatic microsomes from 3-MC- or DBA-treated fish was inhibited by *in vitro* addition of 7,8-benzoflavone (10^{-4} M) (Table 3), as previously shown for rats (Wiebel and Gelboin, 1975).

Aliphatic hydrocarbons are an important quantitative constituent of crude oils. Consequently, we tested the effect of hexadecane administration on xenobiotic-metabolizing enzymes. Treatment of sheepshead with hexadecane (2 x 20 mg/kg, i.p. on Days 1 and 3) did not affect their hepatic microsomal mixed-function oxidase activities, when assayed on Day 8.

TABLE 2. EFFECT OF 3-METHYLCHOLANTHRENE ADMINISTRATION ON HEPATIC MICROSOMAL ENZYMES IN SOME MARINE FISH

SPECIES Treatment	CYTOCHROME P-450[a] CONTENT	BENZO(a)PYRENE[b] HYDROXYLASE	7-ETHOXYCOUMARIN[c] O-DEETHYLASE	BENZPHETAMINE[c] N-DEMETHYLASE
Sheepshead *Archosargus probatocephalus*[d,e]				
Corn oil (3)	0.35 ± 0.05[f]	5.3 ± 1.1	0.40 ± 0.12	1.15 ± 0.25
10 mg/kg, i.p. Day 1 sacrificed Day 8 (4)	0.51 ± 0.21	23.9 ± 4.8	1.10 ± 0.25	1.25 ± 0.40
Stingray *Dasyatis sabina*[d,g]				
Emulphor (4)	0.48 ± 0.05	0.59 ± 0.12	0.08 ± 0.08	0.32 ± 0.17
2 x 15 mg/kg, i.m. Days 1 and 5 sacrificed Day 14 (4)	0.58 ± 0.08	6.12 ± 1.43	4.46 ± 0.12	0.30 ± 0.02
Southern flounder *Paralichthyes lethostigma*[d,e]				
Corn oil (4)	0.11 ± 0.05	0.18 ± 0.06	not detected[h]	not assayed
2 x 10 mg/kg, i.p. Days 1 and 4 sacrificed Day 8 (4)	0.18 ± 0.10	2.74 ± 2.07	0.12 ± 0.01	not assayed
Little skate *Raja erinacea*[i,j]				
Dimethyl sulfoxide (3)	not assayed	0.84 ± 0.28	0.36 ± 0.16	0.96 ± 0.16
2 x 50 mg/kg, p.o. Days 1 and 3, sacrificed Day 11 (3)	not assayed	6.50 ± 0.24	0.56 ± 0.16	1.99 ± 0.20

[a] Nmole/mg protein.

[b] Fluorescence units/min/mg protein.

[c] Nmole product/min/mg protein.

[d] Florida species.

[e] 3-MC was suspended in corn oil at 20 mg/mℓ. Controls received an equivalent volume of corn oil.

[f] Mean ± S.D.

[g] 3-MC was suspended in Emulphor:acetone:water (2:2:6, by volume) at 15 mg/mℓ. Controls received an equivalent volume of vehicle.

[h] Less than 0.001 nmol/min/mg protein.

[i] Maine species.

[j] 3-MC was dissolved in dimethyl sulfoxide at 50 mg/mℓ. Controls received an equivalent volume of dimethyl sulfoxide.

TABLE 3. EFFECT OF *IN VITRO* ADDITION OF 7,8-BENZOFLAVONE ON HEPATIC MICROSOMAL AHH ACTIVITY IN SHEEPSHEAD AND LITTLE SKATE

7,8-BENZOFLAVONE ADDED (M)	BENZO(a)PYRENE HYDROXYLASE ACTIVITY (F.U./MIN/MG PROTEIN)			
	CONTROL SKATE	DBA SKATE[a]	CONTROL SHEEPSHEAD	3-MC SHEEPSHEAD[b]
0	0.23[c]	5.23	1.37[c]	22.6
10^{-7}	0.23	5.08	1.68	22.1
10^{-6}	0.24	3.88	1.72	24.1
10^{-5}	0.21	3.30	2.66	19.8
10^{-4}	0.73	1.16	5.05	8.6
10^{-3}	0.64	0.79	6.05	8.3

[a] Skates were injected i.p. with DBA (10 mg/kg) on Days 1, 2, and 3 and killed on Day 10.

[b] Sheepshead were injected i.p. with 3-MC (20 mg/kg) on Day 1 and killed on Day 9.

[c] Results shown are from a single experiment, which was repeated twice with similar results.

Having obtained significant increases in hepatic AHH activity with all species tested following polycyclic hydrocarbon treatment, we further characterized 3-MC induction in sheepshead. The dose-response of hepatic AHH induction in sheepshead (sacrificed on Day 8), after various i.p. doses of 3-MC on Day 1, is shown in Figure 1. Doses as low as 1 mg/kg caused hepatic AHH activity to double, but this activity was virtually unaffected (neither stimulated nor inhibited) by _in vitro_ addition of 7,8-benzoflavone. At all higher doses, hepatic AHH activity of treated fish was inhibited by _in vitro_ addition of 7,8-benzoflavone. 3-MC doses above 10 mg/kg did not increase the extent of induction at the time the experiment was performed (December and January). Higher treated/control AHH activity ratios were obtained with groups of sheepshead dosed in March (2 x 20 mg/kg) or June, July, and August (10 mg/kg), although the actual activities (F.U./min/mg of protein) in hepatic microsomes from control fish were similar in summer (4.5 ± 3.2, mean ± S.D., n=20) and winter (4.2 ± 1.5, n=19). Control values varied up to 5-fold between different groups of fish, although AHH activity in almost all of the control fish was stimulated by _in vitro_ addition of 7,8-benzoflavone.

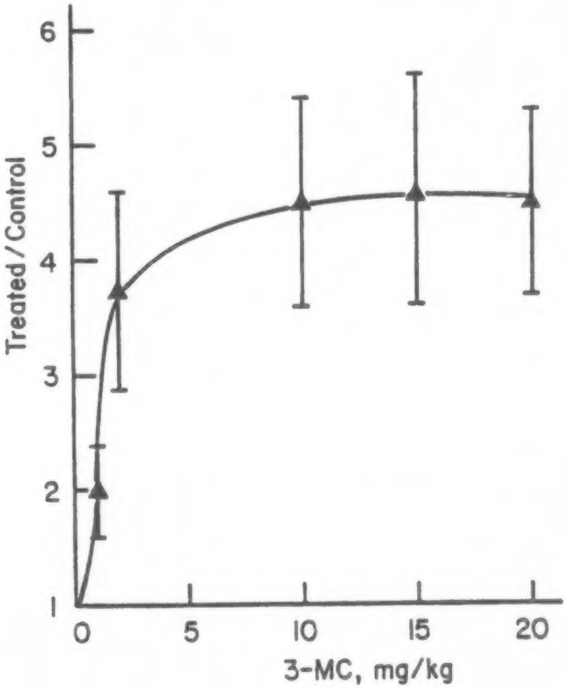

Figure 1. Dose response of AHH induction in sheepshead hepatic microsomes.

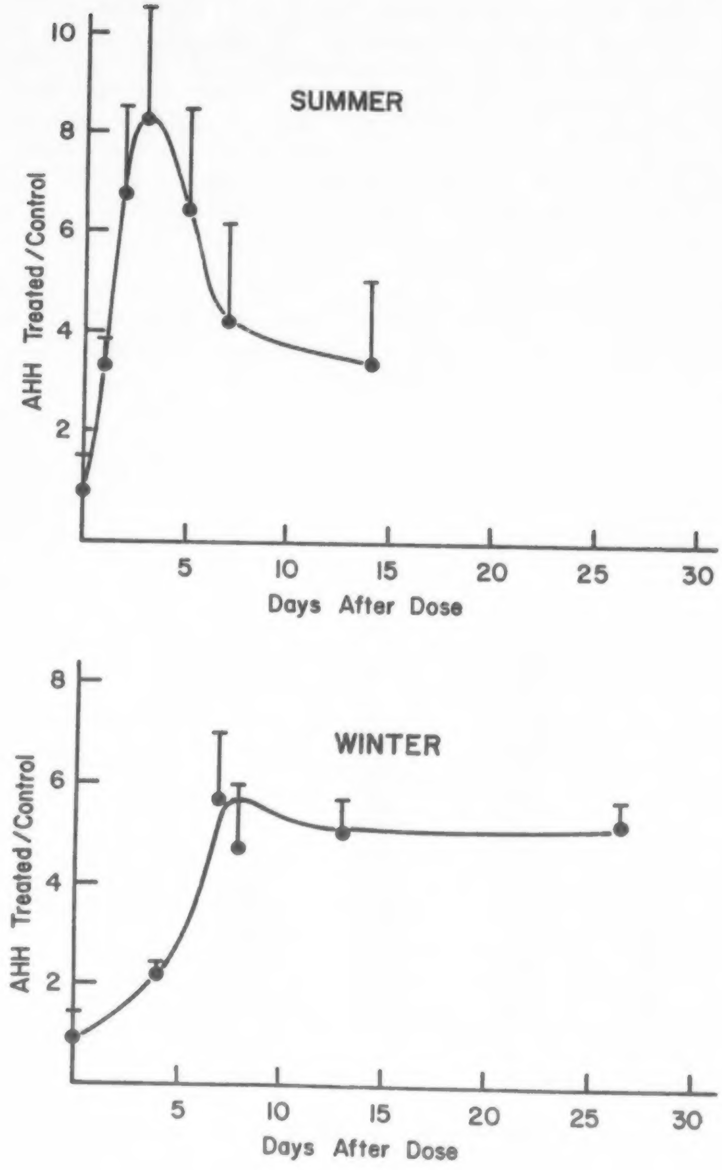

Figure 2. Effect of season on the time course of induction of hepatic microsomal AHH activity in sheepshead.

Since the ocean temperature in St. Augustine, FL, varies from 11° in January to 28° in August, we studied the time course of induction at both seasons. Mixed-function oxidase activities were assayed in hepatic microsomes from groups of sheepshead sacrificed at different times after single doses of 20 mg/kg 3-MC (winter) and 10 mg/kg 3-MC (summer). In preliminary experiments, 20 mg/kg 3-MC was found to be relatively toxic during summer; consequently, the lower dose (10 mg/kg) was used. The results for AHH activity are shown in Figure 2. In summer, maximum induction (8.5-fold) was found 72 hr after administration of 3-MC (Day 4). Hepatic microsomal AHH activities in fish sacrificed only 24 hr after injection of 3-MC (Day 2) were 3.5-fold higher than in controls, and 7,8-benzoflavone inhibited AHH activities in these treated fish. In winter, fish sacrificed 96 hr after the dose (Day 5) were induced only 2-fold, and their AHH activities were stimulated by addition of 7,8-benzoflavone. AHH activities in fish sacrificed on Days 8 to 40, after a dose of 3-MC on Day 1, were inhibited by 7,8-benzoflavone and were induced to similar extents (4- to 5-fold) throughout this period. By Day 63, AHH activities were down to 1.8 times control values; 7,8-benzoflavone stimulated activity in only 1 of 3 treated fish assayed at this time.

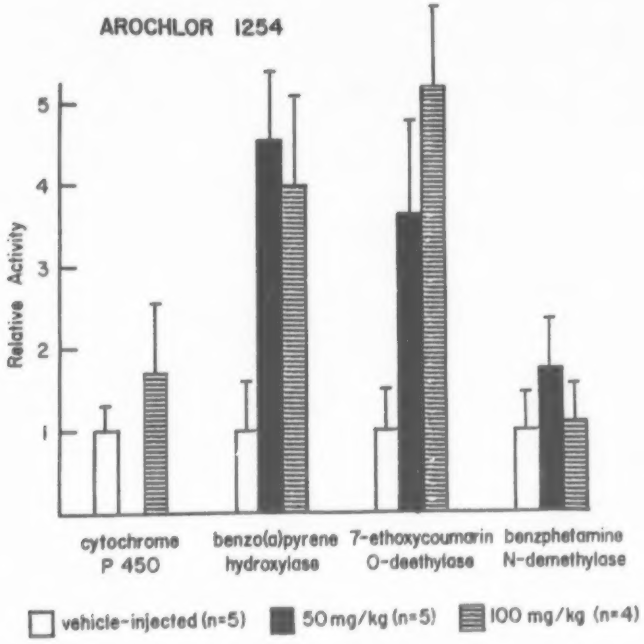

Figure 3. Effect of arochlor 1254 on sheepshead hepatic microsomal mixed-function oxidase activities.

Effects on hepatic epoxide hydrase and glutathione S-transferase activities - None of the aromatic hydrocarbons induced hepatic EH or GSH-T activities with any epoxide substrate in any of the species studied. However, in several experiments with sheepshead, usually those in which high doses were used, EH activity was depressed in treated fish to about 75% of the activity in control fish. For any one experiment, the depression was seldom statistically significant, due to the high variation between individuals; but since the depression occurred repeatedly, it can probably be attributed to the PAH treatment. A similar depression of EH activity was found in hepatic microsomes from stringrays treated with 3-MC (2 x 15 mg/kg, i.m.). EH activity in 3-MC-treated rays was 4.8 ± 1.2 nmol/min/mg protein (mean=S.D., n=4) and activity in controls was 7.2 ± 2.1 (mean ± S.D., n=4). Hepatic GSH-T activity was not depressed or elevated in any of the hydrocarbon-treated fish; however, a small depression of activity occurred in 3-MC-treated sting ray. GSH-T activity in hepatic cytosol from 3-MC-treated rays was 5.22 ± 0.99 nmol/min/mg protein (mean ± S.D., n=4); and activity in controls was 6.75 ± 0.24 (mean ± S.D., n=4).

Polyhalogenated Hydrocarbons

Effects on mixed-function oxidation -- Mixtures of PCBs (Aroclor 1254) and PBBs (Firemaster FF1) both caused induction of AHH and 7-EC activities in sheepshead after i.p. injection (Figures 3 and 4). These mixtures also slightly induced cytochrome P-450 content and BND activities in livers of treated fish, but again the increase was not always statistically significant. Induction with Aroclor 1254 was achieved with doses of 50 and 100 mg/kg, i.p. The effects of lower doses were not studied. In an initial experiment with Firemaster FF1 at a dose of 50 mg/kg, two of five sheepshead died within five days, at which time the remaining fish had induced AHH and 7-EC activities and cytochrome P-450 content. A more thorough investigation of the effects of Firemaster FF1 on hepatic xenobiotic-metabolizing enzymes was carried out with a dose of 15 mg/kg. The results of experiments conducted in winter are summarized in Figure 4. Peak induction of hepatic AHH and 7-EC activities was observed at Day 20 for fish dosed on Day 1; however, at Days 28 and 56, enzyme actiity with these two substrates was still induced. Experiments in summer in which fish sacrificed on Days 28 and 56 after an identical dose of Firemaster FF1 on Day 1 produced different results. In summer, Firemaster FF1-treated sheepshead sacrificed on Day 28 showed 16-fold induction of AHH, 5-fold induction of 7-EC, and 2-fold induction of BND in hepatic microsomes compared with vehicle-injection controls, whereas sheepshead sacrificed on Day 56 had only 1.7-fold induction of AHH, 2-fold induction of 7-EC, and no induction of BND compared with controls.

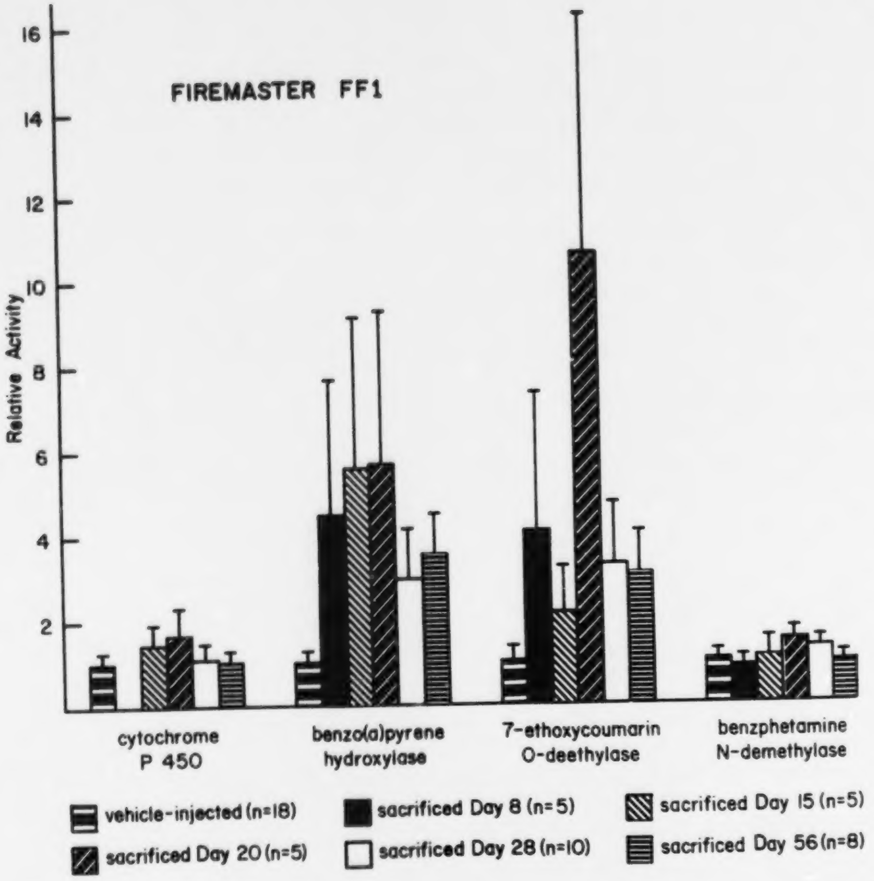

Figure 4. Effect of Firemaster FF1 on sheepsheed hepatic microsomal mixed-function oxidase activities.

The effects of a single dose of 3,3'4,4'5,5'-HCB (10 mg/kg) on hepatic mixed-function oxidation in sheepshead are shown in Figure 5. AHH activities in treated fish were double those of control values by Day 4 and remained elevated at least until Day 24. However, hepatic microsomal AHH activity in sheepshead treated with 3,3'4,4'5,5'-HCB was stimulated by the *in vitro* addition of 10^{-4} M 7,8-benzoflavone, whereas hepatic AHH activity from sheepshead treated with PAH or mixtures of PCBs and PBBs was inhibited by 10^{-4} M 7,8-benzoflavone. 7-EC activity was induced to about the same extent as AHH activity in 3,3'4,4'5,5'-HCB-treated fish. In addition, cytochrome P-450 content, BND activity, and NADPH-cytochrome *c* reductase activity were increased 2-fold over control values in treated sheepshead sacrificed on Day 15.

TABLE 4. EFFECT OF POLYHALOGENATED BIPHENYL ADMINISTRATION ON HEPATIC MICROSOMAL EPOXIDE HYDRASE ACTIVITY

SHEEPSHEAD

Date sacrificed (month/year)	Dose mg/kg	Time Sacrificed (dose given on Day 1)	EPOXIDE HYDRASE ACTIVITY[b] Treated	Control	Ratio Treated/Control
AROCHLOR 1254					
3/77	100	7	4.8 ± 1.5 (4)[c]	5.2 ± 1.3 (3)	0.92
4/77	50	7	5.1 ± 0.7 (4)	2.4 ± 0.6 (3)	2.13
3,3',4,4',5,5'-HEXACHLOROBIPHENYL					
6/78	10	4	5.9 ± 1.5 (5)	4.9 ± 1.1 (3)	1.20
4/78	10	9	7.4 ± 1.1 (5)	4.5 ± 2.1 (3)	1.64
5/78	10	15	7.9 ± 1.6 (4)	4.6 ± 0.6 (3)	1.72
5/78	10	24	7.9 ± 3.0 (3)	5.2 ± 0.1 (3)	1.52
FIREMASTER FF1					
10/77	15	8	6.7 ± 1.9 (5)	6.9 ± 1.4 (3)	0.97
1/78	15	15	5.0 ± 1.3 (5)	4.6 ± 1.7 (3)	1.09
1/78	15	20	4.3 ± 1.3 (5)	2.7 ± 0.4 (3)	1.59
1/78	15	28	6.1 ± 0.8 (5)	5.0 ± 3.6 (3)	1.22
8/77	15	28	5.1 ± 0.6 (5)	3.4 ± 0.9 (3)	1.50
2/77	15	56	4.7 ± 1.0 (5)	5.8 ± 0.2 (3)	0.81
9/78	15	56	7.2 ± 1.5 (5)	6.1 ± 2.1 (4)	1.18
2,2',4,4',5,5'-HEXABROMOBIPHENYL					
3/78	20	17	4.5 ± 1.6 (5)	4.9 ± 1.3 (3)	0.92
3/78	3 x 20	28	5.6 ± 2.4 (5)	5.6 ± 1.3 (3)	1.00
4/78	3 x 20 + 1 x 40	40	5.5 ± 0.5 (5)	5.0 ± 2.0 (3)	1.10

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[a] All compounds were administered by i.p. injection of a corn oil solution or suspension. Controls received corn oil.

[b] Nmoles product formed/min/mg of protein.

[c] Mean ± S.D. for (n) individuals.

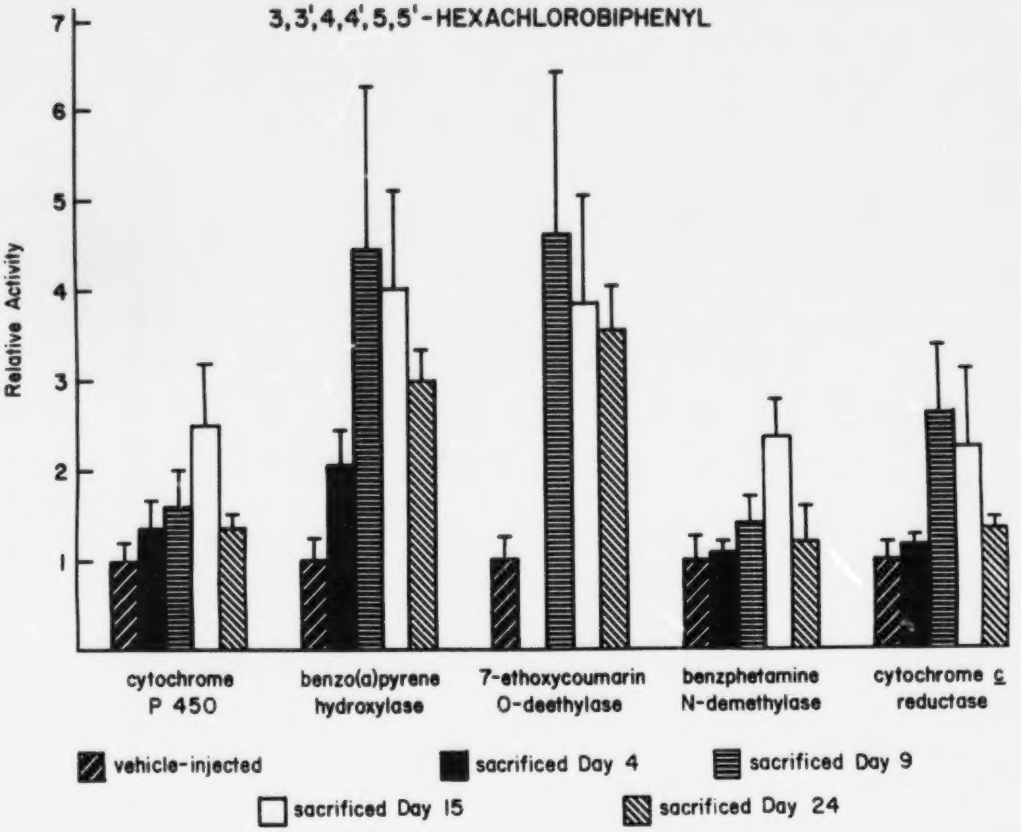

Figure 5. Effects of 3,3'4,4',5,'5-hexachlorobiphenyl on sheepshead hepatic enzymes.

Multiple doses of 2,4'4,4',5,5'-HBB had no effect on any of the mixed-function oxidase enzymes assayed (Figure 6). This isomer was relatively nontoxic, in contrast to the Firemaster FF1 mixture, and no injected sheepshead died. 2,2',4,4',5,5'-HBB accounts for about 60% of Firemaster FF1 (Moore et al., 1978).

Effects on hepatic epoxide hydrase and glutathione S-transferase activities--Aroclor 1254 appeared to have an effect on EH activity in sheepshead treated with 50 mg/kg but no effect was observed with 100 mg/kg (Table 4). However, this appeared to be due only to the lower EH activities in the control fish of the 50 mg/kg group. For example, the mean specific EH activity of all sheepshead assayed from March 1976 to August 1978 was 5.48 ± 2.14 (144) (mean ± S.D., [n]). The effect of 3,3'4,4'5,5'-HCB administration was more consistent. Mean EH activity of treated sheepshead was increased at each time period studied, compared with mean control activity, and the difference was statistically significant ($p < 0.05$) for sheepshead sacrificed on Day 15.

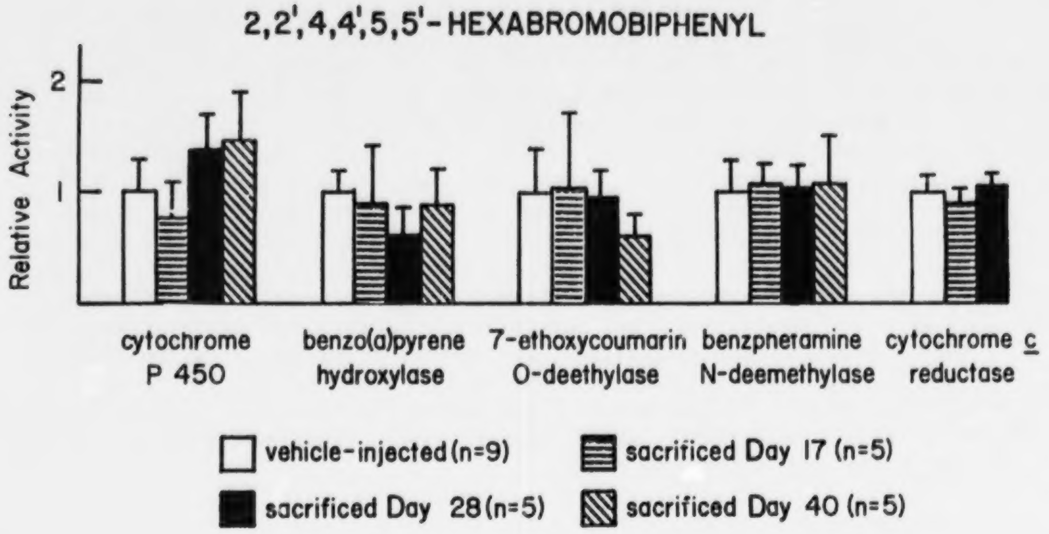

Figure 6. Effect of 2,2',4,4',5',5,-hexabromobiphenyl on sheepshead hepatic enzymes.

Firemaster FF1-treated fish sometimes had higher EH activities than controls, but the difference was significant ($p < 0.05$) only in one group of fish. In our experiment, mean EH activity in control sheepshead was also lower than usual. 2,2'4,4'5,5'-HBB administration had no effect on EH activities. None of the treatments with halogenated biphenyls caused a significant change in hepatic cytosolic fraction GSH-T activity with styrene oxide, except Aroclor 1254 at 50 mg/kg, which again appeared to be related to lower-than-normal specific activities in the control fish.

DISCUSSION

Polynuclear aromatic hydrocarbons--Polynuclear aromatic hydrocarbons such as 3-MC, DBA, and DMBA have an effect on hepatic microsomal mixed-function osidase activity in several species of marine fish, which is similar to the effect PAHs have on hepatic microsomal mixed-function oxidase activity in the rat, guinea pig, and several strains of mice. The lack of induction of EH and GSH-T, and occasional inhibition of EH by PAH, are not inconsistent with the results found in mammals. Induction of both of these enzymes by 3-MC in mammals was usually less than 2-fold; some dose regimes caused inhibition of EH activity in rats (Bresnick *et al*., 1977; Oesch *et al*., 1973). Inhibition of xenobiotic-metabolizing enzymes soon after treatment with inducing agents is a commonly observed phenomenon (Fouts, 1970). In pretreatment studies with wild species, such as fish, it is often difficult to obtain statistically significant changes of low magnitude, such as found for EH and GSH-T activities in livers of PAH-treated inbred rats, due to the variation in enzyme activities.

Differences observed in PAH induction of hepatic microsomal AHH activity in fish vs. mammals relate mainly to the effect of season on the duration of the induction by 3-MC and the sensitivity to 3-MC induction. In summer, induction of sheepshead hepatic microsomal AHH activity is fairly rapid, and peak enzyme activities are attained 72 hr after administration of the dose, after which time a gradual decline in AHH activity is observed. A similar pattern is observed in rats treated i.p. with 80 mg/kg 3-MC (Boobis et al., 1977). In winter, hepatic AHH induction in the sheepshead is not maximal until at least Day 8 after injection of 3-MC, and activities are still elevated to about the same extent on Day 40. Hepatic microsomal AHH activity in the sheepshead was induced by doses as low as 1 mg/kg, i.p., whereas rats are not induced by doses of 10 mg/kg (Boobis et al., 1977). We have found that sheepshead held in a floating dock in the intracoastal waterway had induced hepatic AHH activities after 3 to 4 weeks and that AHH activities are inhibited by in vitro addition of 7,8-benzoflavone. This increase in AHH activity may be due to low concentrations of PAH in the water since barnacles attached to the floating cages contained phenanthrene, methylphenanthrene, fluoranthrene, and pyrene in the parts per billion (ppb) range.

Induction of AHH, 7-EC, and ERF activities with no effect or inhibition of EH and GSH-T activities raises the possibility that PAH-exposed fish may metabolize PAH to toxic intermediates more rapidly than they are detoxified. It is possible that the pattern of metabolites formed in induced fish will be different from those formed in controls and also that the tissue distribution, rates, and routes of excretion will be altered in induction. Other factors such as water temperature may also affect the disposition of xenobiotics in marine species. For species used for human food, it is important to know the tissue distribution of xenobiotics and whether they are stored as metabolites. These factors may also affect the toxicity of a chemical to fish.

A report by Statham and coworkers (1978) showed that benzathracene-induced trout excreted more 2-methylnaphthalene (as metabolites) into bile than did control fish, and that livers of induced fish initially took up more methylnaphthalene than livers of control fish. However, there was little difference between control and induced fish with respect to blood and muscle levels of methylnaphthalene. Further studies are needed in this area to clarify the effect of induction on overall disposition of xenobiotics and toxicity in marine species.

Polyhalogenated biphenyls--The effects of mixtures of PCBs and PBBs on hepatic microsomal mixed-function oxidase activities in sheepshead are generally similar to those found in mammals. Statistically significant increases in EH activities were found only in one group of Aroclor 1254- and one group of Firemaster FF1-treated sheepshead, and enzyme activities in control fish for both of these experiments were unusually low. Analysis of EH data obtained in 18 months (from March 1976 to December 1977) showed that EH tended to be lower between March and August, especially in female sheepshead. However, data obtained so far in 1978 are not consistent with earlier observations and provide no explanation for the wide variation of

EH activity in control sheepshead (activities measured in control sheepshead ranged from 1.85 to 18.85 nmol/min/mg of protein), other than genetic heterogeneity.

The two single polyhalogenated biphenyl isomers studied did not appear to have the same effects in sheepshead as those found in mammals. 3,3',4,4'5,5'-HCB was found to be a polycyclic hydrocarbon-type inducer in rats (Goldstein et al., 1977), but the effect of this isomer in sheepshead hepatic ensymes was not identical to the effect of 3-MC. 3,3'4,4'5,5'-HCB-treated sheepshead had elevated AHH, 7-EC, BND, and cytochrome c reductase activities and cytochrome P-450 content in hepatic microsomes, especially on Days 9 and 15 after a dose of 10 mg/kg. However, 7-8,benzoflavone did not depress the AHH activities of treated fish. EH activity was also significantly higher in 3,3'4,4'5,5'-HCB-treated fish on Day 15.

2,2'4,4'5,5'-HBB was without effect on sheepshead hepatic enzymes, even at the highest doses tested, although it is a phenobarbital-type inducer in rats (Moore et al., 1978). We wanted to study the effect of this PBB isomer on sheepshead since it is the major compound present in Firemaster FF1, which induced xenobiotic metabolism in sheepshead. These experiments suggest that the induction caused by Firemaster FF1 may be due to a minor component, or minor components, of the mixture. This lack of a phenobarbital-like induction has been previously reported in fish (Bend and James, 1978).

The doses of both PAH and polyhalogenated biphenyls which induced AHH and 7-EC activities in sheepshead were lower than those needed to effect induction in the rat (Brensnick et al., 1977; Dent et al., 1976; Goldstein et al., 1977). Since sheepshead and other fish exposed environmentally to very low levels of PAH pollution are often found to have induced AHH (Payne, 1976), 7-EC, and ERF activities, and sometimes other hepatic enzyme activities, it appears possible that some species of fish are very sensitive to induction by PAH. The results obtained with PCBs and PBBs suggest that the same is true for these chemicals. Thus, it is important to find out how induction of hepatic enzymes affects the rate of metabolism of pollutant chemicals in fish and to determine if induction of the hepatic mixed-function oxidase systems of fish can be used as a sensitive indicator for the presence of toxic pollutants that behave as polycyclic hydrocarbon-like inducers in the aquatic environment.

ACKNOWLEDGMENTS

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METABOLISM OF BENZO(a)PYRENE BY *CIONA INTESTINALIS*

by

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ABSTRACT

Some ascidians (sea squirts) such as *Ciona intestinalis* thrive in ports and dock regions where they are exposed to polycyclic aromatic hydrocarbons. To determine if *Ciona intestinalis* can metabolize such hydrocarbons, specimens were exposed to $[G\text{-}^3H]$benzo(*a*)pyrene (BaP) (0.5 nmole/mℓ seawater) for 24 hr, and the seawater and several tissues were analyzed for BaP and BaP metabolites. The BaP was concentrated by the organisms; large animals contained as much as 60% of the BaP after 24 hr. Samples of all ascidian tissues and of seawater from the ascidian-containing beakers contained slightly higher proportions of water-soluble radioactive derivatives than did samples from control beakers. An unidentified BaP derivative with chromatographic properties similar to those of BaP-dihydrodiols was detected in the seawater from the ascidian-containing beaker. Protein-associated radioactivity was found in several ascidian tissues and was highest in the intestine. These results suggest that *Ciona intestinalis* is able to concentrate a polycyclic hydrocarbon present in the water, and that the hydrocarbon is slowly lost from the organism as a water-soluble derivative. Whether this is due to spontaneous breakdown of the hydrocarbon or to a low level of hydrocarbon-metabolizing activity of the organism has not been established, but the very slow rate of conversion of the hydrocarbon to water-soluble forms probably serves to protect the organism from biological effects induced by hydrocarbons in organisms with high hydrocarbon-metabolizing activity.

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INTRODUCTION

Several polycyclic aromatic hydrocarbons found in petroleum products or combustion products induce cytotoxicity, mutation, and transformation of cells in culture and cancer in a number of mammalian species (NAS, 1972; Dipple, 1976; Freudenthal and Jones, 1976, 1978). Metabolism of the inert hydrocarbon molecule is required for the induction of such biological effects. Only a few metabolic pathways lead to the formation of those metabolites that are more active than the parent hydrocarbons in inducing biological effects, and most pathways lead to detoxification of the hydrocarbons through the formation of oxidized derivatives and their conjugates (NAS, 1972; Heidelberger, 1973; Sims and Grover, 1974; Dipple, 1976; Freudenthal and Jones, 1976, 1978; Jerina and Daly, 1976; Diamond and Baird, 1977).

The Ascidiacea (subphylum: Tunicata), or sea squirts, are a class of filter-feeding marine organisms (Millar, 1953, 1971); some species are able to thrive in areas containing various pollutants (Papadopoulou and Kanias, 1977; Riggio and Mazzola, 1976). We have observed that *Ciona intestinalis*, a species frequently used for studies in embryology because of the determinate cleavage pattern of its eggs, thrives in dock regions where oil pollution exists. Polycyclic aromatic hydrocarbons are frequently found in such areas (Kraybill, 1976). Some large adult *Ciona* collected in the area of Sandwich, MA; relativley large amounts of petroleum residues on their surfaces observed showed no apparent toxicity from this exposure.

To find out if *Ciona intestinalis* can thrive in areas containing polycyclic aromatic hydrocarbons by metabolizing them through pathways that do not form biologically active metabolites, the metabolism of benzo(a)pyrene (BaP), a widespread environmental contaminant, was examined with techniques developed for the analysis of hydrocarbon metabolism in tissue culture. These include treatment with an isotopically labeled hydrocarbon, enzymatic cleavage of conjugated metabolites, isolation of metabolites by organic solvent extraction, and analysis by chromatography (Diamond *et al*., 1967; 1968; Duncan *et al*., 1969; Sims, 1970; Huberman *et al*., 1971; Baird and Brookes, 1973; Cohen *et al*., 1976; Baird *et al*., 1977, 1978; Selkirk, 1978; Philips and Sims, 1979).

MATERIALS AND METHODS

Animals--Specimens of *Ciona intestinalis* (L.) were collected off Cape Cod, MA, shipped in seawater to the Wistar Institute, and maintained 7 to 14 days in an Instant Ocean tank (Aquarium Systems, Inc., East Lake, OH) of artificial seawater at 15° C. At least 1 hr before experimental use, animals were transferred to glass beakers (one animal per beaker) containing 50 mℓ seawater and 20 μg rifampicin/mℓ and maintained at 18° C throughout the experiment. Animals approximately 4 cm in length were classed as large; those approximately 1.5 cm in length as small.

Benzo(a)pyrene metabolism--(G-^{3}H)benzo(a)pyrene (Sp. Act. 3-13 Ci/nmole) was purchased from Amersham Corp., IL, and dissolved in dimethyl sulfoxide immediately before use. All studies were carried out in the presence of yellow light or in the absence of light to prevent photodecomposition of the BaP. The BaP solution was added to the seawater to give final concentrations of 0.5 nmole BaP/mℓ and 0.2% dimethyl sulfoxide. Immediately after the BaP was added to the beaker, a 2 mℓ sample of water was removed for incubation as the control. After 24 hr of incubation, samples of water were removed for analysis from both the ascidian-containing and control beakers, the animals dissected, and the tissues frozen for metabolite analysis.

BaP metabolites and unchanged BaP were extracted from water and tissue samples by a two-step chloroform:methanol:water procedure and the amount of radioactivity in each phase determined by liquid scintillation counting of 0.1 mℓ aliquots (Baird and Diamond, 1976). The material extracted by the organic solvent was analyzed by high-pressure liquid chromatography (HPLC) (Selkirk, 1978) or thin-layer chromatography (TLC) (Baird et al., 1978; 1979). HPLC analyses were carried out on an Altex Model 312 using a 4.6 mm x 25 cm Spherisorb 5 μm ODS reversed-phase column at 30° C. Samples were eluted at a flow rate of 1 mℓ/min with a 60 min concave gradient (Altex exponent 3) from 3:2 to 4:1 methanol-water followed by 10 min at 4:1; 140 fractions (0.5 min) were collected and radioactivity was measured by liquid scintillation counting (Baird et al., 1979). Elution positions of markers of BaP metabolites were determined by UV-absorbance. TLC analyses were carried out on Eastman 13179 silica gel chromogram sheets without fluorescent indicator and developed in benzene:ethanol (19:1) or toluene: ethanol (19:1). The samples were then cut into 1 cm squares, eluted with 1 mℓ methanol, and measured by liquid scintillation counting (Baird and Diamond, 1976). Markers of BaP metabolites were chromatographed on each sheet and visualized by short-wave UV light.

Protein-associated radioactivity--Tissues to be analyzed were first homogenized in distilled water. BaP and its metabolites were extracted with chloroform:methanol:water as described. The protein interface was removed, extracted three times with methanol and three times with ethyl acetate, and dried with nitrogen. The interface was then dissolved in 0.5 N sodium hydroxide solution, neutralized with 1 N hydrochloric acid, and extracted with two volumes ethyl acetate. The interface from this extraction was removed, extracted with methanol (three times) and ethyl acetate (three times), and dried under nitrogen. The sample was dissolved in 0.5 N sodium hydroxide solution and neutralized with hydrochloric acid. Aliquots were removed for analysis of radioactivity by liquid scintillation counting and of protein by a modification of the Lowry procedure (Ross and Schatz, 1973).

RESULTS

A large ascidian was exposed to (^{3}H)BaP (0.5 nmole/mℓ water) for 24 hr, then an aliquot of the water was extracted with chloroform and methanol, and the chloroform-extractable material was analyzed by HPLC. The HPLC elution profiles of the chloroform extracts of water from a control beaker (Figure 1a) and the ascidian-containing beaker (Figure 1b) show that extracts contained mainly unchanged BaP. The sample from the ascidian water, however, contained an unidentified peak, designated "unknown No. 1," that eluted slightly later than the BaP-9,10-diol markers. With TLC analysis, a similar amount of radioactivity showed an R_F comparable to markers of BaP-diols. Both ascidian and control water samples contained similar small amounts of BaP-quinones.

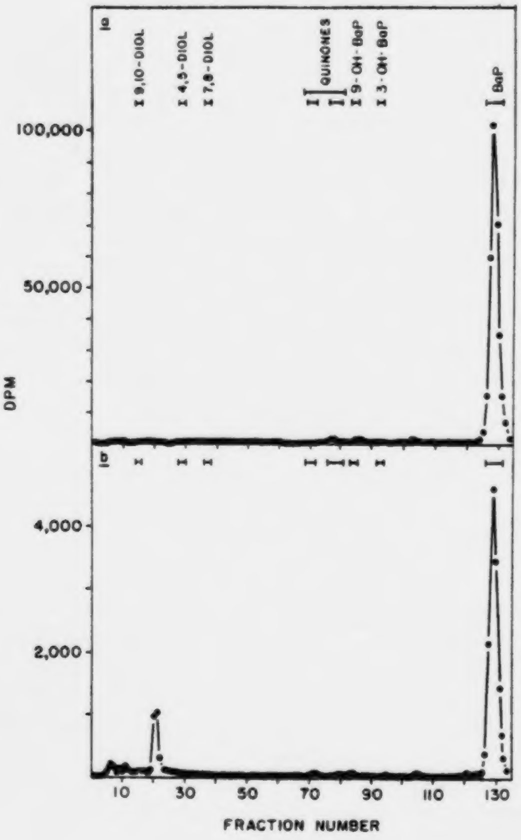

Figure 1. HPLC elution profiles of BaP and BaP metabolites in seawater from a control beaker with no ascidian (a) and a beaker containing a large *Ciona intestinalis* (b). (^3H)BaP was added to a beaker containing an ascidian and a sample of water was immediately removed and incubated separately as a control. After 24 hr, 0.2 mℓ samples from each beaker were extracted with chloroform:methanol:water and the chloroform extracts analyzed by HPLC, as described in *Materials and Methods*. The elution positions of BaP metabolite markers are shown at the top of each sample. ● DPM/0.5 mℓ fraction.

TABLE 1. RADIOACTIVITY REMAINING IN SEAWATER AFTER EXPOSURE OF ASCIDIANS TO (^{3}H)BaP

Sample	% of radioactivity not extractable by organic solvent	Peaks as % of radioactivity extractable by organic solvent		
		Unknown #1	Quinones	BaP
Large Ascidian	26	15	5	80
Control	1	0.2	3	96
Small Ascidian	1.5	--	4	93
Control	0.6	--	4	95

After exposure of ascidians to (^{3}H)BaP (0.5 nmole/mℓ water) for 24 hr, water samples from beakers containing either large (4 cm) or small (1.5 cm) ascidians and from control beakers were extracted with choloroform:methanol: water and the chloroform phases analyzed by HPLC. Results are averages of two or three experiments.

TABLE 2. RADIOACTIVITY RECOVERED FROM ASCIDIAN TISSUES AFTER EXPOSURE TO (^{3}H)BaP

Sample	% of radioactivity not extractable by organic solvent	Peaks as % of radioactivity extractable by organic solvent			Protein-associated radioactivity
		Unknown #1	Quinone	BaP	pmoles [^{3}H]BaP/mg protein
Control	1	0.2	3	96	—
Tunic	6	0.2	4	95	6
Intestine	2	0.2	4	95	10
Branchial basket	2	0.3	2	97	5
Ovary	ND[a]	ND	ND	ND	2

[a] ND = not determined. Large ascidians were exposed to (^{3}H)BaP for 24 hr and dissected. The BaP metabolites in each tissue were extracted and analyzed by HPLC as described in Table 1. Water from a beaker containing no ascidian was used as a control. All results are the average of two experiments.

The most striking difference between ascidian water and control water was in the amount of radioactivity remaining after 24 hr. Control water contained nearly 70% of the radioactivity added, in contrast to the less than 7% in the water samples from large ascidians. Thus, most of the BaP had been removed by the ascidian. This BaP removal was dependent upon the size of the ascidian, for only a small amount (5 to 10%) was removed by small ascidians. The water from the small ascidian beakers contained mainly unchanged BaP, with small amounts of BaP quinones (Table 1). The amount of BaP that precipitated in the water was similar in all cases: an acetone rinse of beakers after removal of the water resulted in the recovery of 20 to 25% of the radioactivity from beakers both with animals and without.

Tissues were dissected from two large ascidians and analyzed by the HPLC procedure to determine if BaP metabolites had been formed but not released into the water (Table 2). No identifiable BaP metabolites were detected in the three tissues examined. Quinones were present in all tissues, but the amounts were not significantly greater than those found in water from the control beaker. All tissue samples contained more water-soluble radioactivity than the control, but the nature of this water-soluble material could not be determined. In the large ascidians, several times as much radioactivity was recovered from the intestine as from the branchial basket. In the small ascidians, the material recovered from each tissue was similar to that of the large ascidian except that more was recovered in the branchial basket than in the intestine (data not shown).

To see if any hydrocarbon metabolites that formed interacted with cellular components, we measured the amount of protein-associated radioactivity (Table 2). After the tissues were exhaustively extracted by the procedure described in Materials and Methods to remove any unbound BaP or BaP metabolites, the protein-associated radioactivity ratios were calculated (Table 2). Although the amounts were low, BaP appeared to become bound to cellular proteins, especially in the intestine.

DISCUSSION

We have shown that the ascidian, *Ciona intestinalis*, can remove the hydrocarbon BaP from seawater. The amount removed by large ascidians was greater than that removed by small ascidians, but the concentration of BaP within the organism was greater than in the surrounding water.

No identifiable BaP metabolites were detected in either the surrounding water or in any of the tissues examined. Some BaP quinones were found, especially in the intestine where a large portion of the BaP had accumulated, but the ratio of quinones to BaP did not differ from that in the control seawater. These quinones may represent a spontaneous breakdown products of BaP. However, three lines of evidence suggest that ascidians possess a low level of BaP metabolizing activity: (1) An unidentified BaP derivative with chromatographic properties similar to those of a BaP-diol was recovered in the water from the beaker that contained the large ascidian, but this material was not present in the control water; (2) A higher percentage of the radioactivity was recovered in the water phases of chloroform- methanol extractions of tissue samples and water from the beaker containing the ascidian than from the control water sample; (3) Protein-associated radioactivity was recovered in several tissues and was greatest in the tissue with the highest BaP concentration.

These findings suggest that *Ciona intestinalis* is capable of concentrating hydrocarbons such as BaP from seawater. BaP is then slowly converted to a water-soluble form, either by breakdown, by bacterial metabolism, or by a low metabolizing activity of the organism. A small portion of the hydrocarbon also becomes bound to tissue components.

The very low level of hydrocarbon metabolism probably protects *Ciona* from the induction of toxicity by such compounds; although most of the hydrocarbon added to the water was concentrated in the organism, the ascidian metabolized only a small amount in 24 hr. In contrast, cultures of rodent cells exposed to similar concentrations of hydrocarbons would have metabolized almost all of the hydrocarbon within 24 hr (Baird *et al*., 1977). Thus, *Ciona* may be able to gradually remove the hydrocarbons it accumulates without generating toxic levels of reactive metabolites. The finding that some BaP becomes bound to cellular components warrants further investigation. Although the major DNA-binding derivative in several mammalian systems has been identified as a dihydrodiol-epoxide of BaP (Weinstein *et al*., 1976; Koreeda *et al*., 1978; Phillips and Sims, 1979), there is evidence that BaP-quinones may be involved in BaP-DNA interactions in microsomal systems (Pelkonen *et al*., 1978). BaP-quinones are present in the tissue of BaP-treated *Ciona* and therefore may be involved in BaP-macromolecule interactions in this organism.

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BIOACTIVATION OF POLYNUCLEAR AROMATIC HYDROCARBONS TO CYTOTOXIC AND MUTAGENIC PRODUCTS BY MARINE FISH

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ABSTRACT

Levels of hepatic cytochrome P-450 and mixed-function oxygenase activity differed markedly between marine fish species scup, *Stenotomus versicolor*, and winter flounder, *Pseudopleuronectes americanus*, and between male and female winter flounder. Hepatic preparations from all these fishes, however, were capable of efficiently activating carcinogenic polynuclear aromatic hydrocarbons to mutagenic derivatives. The results indicate that coastal marine fishes may be at a risk to carcinogenic aromatic hydrocarbons in marine sediments.

INTRODUCTION

Polynuclear aromatic hydrocarbons are metabolized or biotransformed by microsomal cytochrome P-450 dependent mixed-function oxygenases in tissues of diverse species (Walker, 1978). The metabolism of carcingogenic polynuclear aromatic hydrocarbons by some species is known to result in formation of mutagenic derivatives (Wislocki *et al*., 1976). Studies have indicated that certain metabolites are responsible for mediating the carcinogenic activity of these compounds (Levin *et al*., 1977, 1978; Slaga *et al*., 1977; Hecht *et al*., 1978). However, microsomal cytochromes P-450 from different mammalian tissues, different species, or animals subjected to different treatment appear to vary in their ability to metabolize and activate polynuclear aromatic hydrocarbons to mutagens *in vitro* (Ames *et al*., 1975; Levin *et al*., 1976). Such variation may indicate differences in susceptibility to mutagenic or carcinogenic potential of polynuclear aromatic hydrocarbons.

Microsomal electron transport systems in fish tissues are qualitatively similar to those in mammals (Pohl et al., 1974; Chevion et al., 1977; Stegeman and Binder, 1979). The levels of components of these systems and the mixed-function oxygenase reactions carried out, however, are generally lower in fish (Pohl et al., 1974; Bend et al., 1977). Yet, in some species the activity of hepatic aryl hydrocarbon [benzo(a)pyrene] hydroxylase is normally found to be higher than seen in mammals (Ahokas et al., 1975; Stegeman and Binder, 1979). Data suggest these fish may have cytochrome(s) P-450 that catalytically resemble(s) 3-methylcholanthrene-induced cytochrome P-448 in mammals, whereas others may not (Bend et al., 1977). We do not know whether such apparent functional differences in cytochromes P-450 within or between fish species might be associated with varied capacity to activate polynuclear aromatic hydrocarbons. The present contribution describes aspects of hepatic cytochrome P-450 systems and the in vitro activation of selected polynuclear aromatic hydrocarbons by two species of marine fish, scup (porgy), and winter flounder.

MATERIAL AND METHODS

Chemicals--Benzo(a)pyrene used in enzyme assays was obtained from Aldrich Chemical Company (Milwaukee, WI). Benzo(a)pyrene, 7,12-dimethylbenzanthracene and 1,2,3,4-dibenzanthracene used in mutation assays were obtained from Sigma Chemical Co. (St. Louis, MO). NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, aminopyrine, Tris, and HEPES were obtained from Sigma.

Animals--Adult male and female scup, *Stenotomus versicolor*, about 100 to 200 g, were collected by angling in Great Harbor, Woods Hole, MA, in August 1977; winter flounder, *Pseudopleuronectes americanus*, were obtained in outer Narragansett Bay, RI, by otter trawl in December 1977. Males were 270 to 350 g and females 480 to 520 g. Scup were maintained for four months in 800 gallon tanks at 19° ± 1° C in flowing water filtered through gravel and sand at the National Marine Fisheries Service, Woods Hole, MA. Fish were fed a diet of chopped smelt and clams ad libitum every two days.

Flounder were maintained at ambient temperatures at the U.S. Environmental Protection Agency, Environmental Research Laboratory, Narragansett, RI, prior to transport to Woods Hole. At the time of use, the scup were sexually quiescent, and the winter flounder were fully hydrated, ready to spawn. No fish used in these studies received any experimental treatment.

Tissue preparations--Animals were killed by decapitation. Excised livers were placed immediately in ice-cold 0.1 M phosphate buffer, pH 7.3. Tissues were minced and homogenized in 4 volumes of 0.1 M PO_4 buffer pH 7.3, containing 1.15% KCl and 3 mM $MgCl_2$ by a Potter-Elvehjem tissue grinder with 4 passes of the pestle at 1350 and 4 at 1900 rpm. Post-mitochondrial supernatant (PMS) preparations for use in mutation assays were collected after centrifuging at 9000 xg for 10 min. Microsomal fractions for enzyme assays were isolated from the 9000 xg supernatant as previously described (Stegeman and Binder, 1979). PMS preparations were frozen in liquid N_2 and then held at -80° C until use (up to two months). Enzyme assays on either PMS or microsomes were done immediately.

Enzyme assays--Benzo(a)pyrene hydroxylase and aminopyrine demethylase in hepatic preparations were assayed as previously described (Stegeman and Binder, 1979) by measuring fluorescent product formation and formaldehyde generation, respectively. NADPH-cytochrome c (P-450) reduction and cytochrome P-450 were measured as previously described (Stegeman and Binder, 1979). Protein was determined according to Lowry *et al*. (1951).

Bacterial mutation assays--Reverse mutation assays to histidine prototrophy were carried out with *Salmonella typhimurium* strain TA-98; forward mutation assays to 8-azaguanine resistance (Skopek *et al*., 1978a) employed *S. typhimurium* strain TM-677. The sources and storage conditions for these strains have been indicated (Skopek *et al*., 1978b).

Basic protocols for both the reverse and forward mutation assays have been described (Skopek *et al*., 1978a; 1978b). Exposure to promutagen was in liquid culture in 25 mℓ plastic tissue flasks and 5.0 mℓ volumes that contained an appropriate concentration of bacterial cells, 0.5 mℓ sterile PMS, and 6.5 μmoles NADPH. Hydrocarbons were added to duplicate flasks in 50 μℓ of dimethyl sulfoxide. Flasks were incubated without shaking for 2 hr at 29° C when scup PMS was employed and 25° C when winter flounder PMS was employed. The temperatures selected were near optimal temperatures for benzo(a)pyrene hydroxylase in these two species when assayed over a 2-hr period. Harvest of cells and plating procedures for estimating both bacterial survival and mutation are described elsewhere (Skopek, 1978b). Mutant fractions in both reverse and forward assays are presented as number of mutant clones (x factor)/number of survivor clones plated.

RESULTS

Levels of microsomal electron transport components and mixed-function oxygenase activities in hepatic microsomes were compared in scup, winter flounder, and mice. Results are presented in Table 1. The levels of cytochrome b_5, NADPH- and NADH-cytochrome c reductases in scup were about 20% of those measured in mice. Cytochrome P-450 present in scup was of comparatively greater amounts (about 50% more than observed in mice). However, the Soret absorption maximum of reduced, CO-treated microsomes was about 450 nm in both species. Aminopyrine demethylase activity was also much lower in scup than in mice, but benzo(a)pyrene hydroxylase activity was almost ten-fold greater in the scup.

TABLE 1. HEPATIC MICROSOMAL ELECTRON TRANSPORT COMPONENTS AND MIXED-FUNCTION OXYGENASES IN SCUP, WINTER FLOUNDER, AND MICE

Component	Scup[a]	Winter Flounder		Mouse[a]
		male	female	
	(N ≥ 10)	(N ≥ 3)	(N ≥ 3)	(N ≥ 3)
Liver wt./body wt. %	1.01±0.10[b]	1.02±0.05	2.43±1.7	5.24±0.29
mg mic. protein/g liver	12.4 ±0.5	17.5 ±0.7	23.0 ±2.9	20.0 ±0.32
Cytochrome P-450 nmoles·mg prot.$^{-1}$	0.62±0.08	0.90±0.21	0.19±0.2	1.14±0.12
Cytochrome b_5 nmoles·mg prot.$^{-1}$	0.06±0.02	-	-	0.33±0.03
NADPH-cytochrome c reductase units·mg prot.$^{-1}$ [c]	107± 5	48± 5	38± 5	510± 9
NAPH-cytochrome c reductase units·mg prot. $^{-1}$	183± 6	-	-	913± 59
Aminopyrine demethylase units·mg prot.$^{-1}$	206±34	84±13	51±19	800±151
Benzo(a)pyrene hydroxylase units·mg prot.$^{-1}$	693±40	213±15	77± 5	72± 14

[a] Data from Stegeman and Binder (1979). Mice were adult Charles River CD-1 females.

[b] All values are ± S.E.M.

[c] Units are nanomoles cytochrome c reduced·min^{-1} (reductases), nanomoles HCHO produced normalized to 1 hour (aminopyrine demethylase) and picomoles 3-OH-benzo(a)pyrene equivalents produced·min^{-1} [benzo(a)pyrene hydroxylase].

During spawning, pronounced sex differences in winter flounder were observed in microsomal cytochromes P-450. The Soret absorption maximum of reduced, CO-treated microsomes from male fish was quite clearly at 448 nm rather than 450 nm seen in females, or in scup or mice. The levels of cytochrome P-450 seen in males were more than five times those of females and were greater than those observed in scup. Unlike cytochrome P-450, NADPH-cytochrome c reductase activity was quite similar in male and female flounder, and the levels were lower than in scup. The levels of both aminopyrine demethylase and benzo(a)pyrene hydroxylase activity were also lower in male and female winter flounder than in scup. However, a sexual difference was apparent in the levels of these activities (levels in females were lower than those in males). Benzo(a)pyrene hydroxylase activity in female flounder was almost as low as that seen in mice, while this activity in male flounder was several times greater.

The metabolic activation of benzo(a)pyrene to toxic and mutagenic derivatives by fish liver preparations like those described in Table 1 was initially determined by a reverse mutation assay. Results indicate that untreated scup liver PMS when incubated with NADPH and benzo(a)pyrene was capable of stimulating almost an 85-fold increase in the $\underline{his}^+$ revertant fraction in S. typhimurium strain TA-98. At the same time, there was a 20-fold reduction in survival of S. typhimurium in the complete incubation with 50 μM benzo(a)pyrene. The activation indicated in Table 2 was dependent on the presence of PMS, as well as benzo(a)pyrene and NADPH; a linear dose-dependent increase was observed in both the mutant fraction and the toxicity up to a peak at benzo(a)pyrene concentrations between 40 to 60 μM.

TABLE 2. REQUIREMENTS FOR ACTIVATION OF BENZO(a)PYRENE TO TOXIC AND MUTAGENIC DERIVATIVES IN S. TYPHIMURIUM STRAIN TA-98 BY SCUP LIVER PMS*

Incubation Conditions	Relative Survival	Observed Mutant† Fraction x 10^8
Complete [50 μM B(a)P]	0.05	47.2
Minus B(a)P	1.00	0.55
Minus NADPH	0.91	0.21

*From Stegeman (1977)

†Mutant Fraction refers to the number of *his*$^+$ revertant clones x 10^{-6} per number of survivor clones. Incubation conditions are as described in Materials and Methods.

TABLE 3. ACTIVATION OF POLYNUCLEAR AROMATIC HYDROCARBONS TO MUTAGENIC DERIVATIVES IN S. TYPHIMURIUM STRAIN TM-677 BY SCUP AND WINTER FLOUNDER LIVER PMS

PMS Source (N)[a]	Compound[b]	Concentration[c] (μM)	Relative Survival	Induced Mutant[d] Fraction x 10^5
Scup (9)	B(a)P	40	0.48	85.5
	DBA	36	0.70	32.6
	DMBA	20	0.85	14.6
Winter Flounder				
Male (2)	B(a)P	40	0.73	144.5
Female (2)	B(a)P	40	0.84	79.0

[a] Livers from N fish pooled for PMS preparation.

[b] Abbreviations are B(a)P, benzo(a)pyrene; DBA, 1,2,3,4-dibenzanthracene; DMBA, 7,12-dimethylbenzanthracene.

[c] Data presented have been selected from dose-response curves and represent concentrations at which maximal response was detected, except for female winter flounder. Here the data at 40 μM were selected for comparison with both male winter flounder and scup.

[d] Mutant fraction refers to the number of 8-azaguanine resistant clones x 10^{-4} per number of survivor clones. Background mutant fractions (0 compound) were 4-6(x10^5) within the range previously reported (Shopek *et al.*, 1978b) and have been subtracted.

Activation of polynuclear aromatic hydrocarbons by scup and winter flounder liver PMS was compared, using the forward mutation assay to 8-azaguanine resistance in *S. typhimurium* strain TM-677. The levels of benzo(a)pyrene hydroxylase activity per mℓ of hepatic PMS in the specific preparations used were 4000 pmol 3-OH-benzo(a)pyrene equivalents/min/mℓ for scup, which was twice that in the male winter flounder, 2018 pmol/min/mℓ, and more than six times that in the female, 632 pmol/min/mℓ. The results are presented in Table 3. The mutant fraction resulting from activation of benzo(a)pyrene by scup liver PMS in this assaay was high, as was observed in the *his*$^+$ reversion assay. Scup liver PMS also readily activated 1,2,3,4-dibenzanthracene and 7,12-dimethylbenzanthracene, but the mutant fractions observed were lower than with benzo(a)pyrene.

The mutant fraction induced by activation of 40 μM benzo(a)pyrene by female winter flounder PMS was comparable to that seen with scup. Male winter flounder PMS, on the other hand, was able to induce a somewhat higher mutant fraction than scup with 40 μM benzo(a)pyrene. It is noteworthy that these results are quite different from that seen in levels of benzo(a)pyrene hydroxylase activity in PMS preparations, or found in hepatic microsomal preparations of scup and winter flounder. This suggests that the level of benzo(a)pyrene hydroxylase activity in fish is not a suitable indicator of the capacity to form mutagenic derivatives from a polynuclear aromatic hydrocarbon such as benzo(a)pyrene.

DISCUSSION

Both fish species examined possessed marked ability to metabolically activate polynuclear aromatic hydrocarbons to mutagenic products *in vitro*. The mutant fractions induced with 40 μM benzo(a)pyrene in fact exceeded that [72 (x 10^5)] obtained with 40 μM benzo(a)pyrene and hepatic preparations from Aroclor 1254-induced rats in the forward mutation assay with *S. typhimurium* TM-677 (Skopek *et al*., 1978b). This is particularly interesting because fish used in these studies had received no experimental treatment.

Hepatic preparations from rats uninduced or induced with phenobarbital usually have much lower capacity for activation of benzo(a)pyrene than those from animals treated with 3-methylcholanthrene or mixed inducers such as Aroclor 1254 (Ames *et al*., 1975). This can be attributed to differences in the ability of cytochromes P-450 in the variously treated animals to produce certain mutagenic derivatives of benzo(a)pyrene (Levin *et al*., 1976). By analogy, the ready formation of mutagenic derivates of benzo(a)pyrene by the fish here suggests that cytochromes P-450 in these animals may in some way be catalytically similar to cytochromes P-448 in some mammals. This is consistent with the observation that hepatic benzo(a)pyrene hydroxylase in untreated scup at least is strongly inhibited by 10^{-4} M 7,8-benzoflavone (Stegeman and Binder, 1979), a characteristic of 3-methylcholanthrene-induced cytochrome P-448 in some mammals (Weibel *et al*., 1971). However, it is questionable whether these are features of constitutive cytochromes P-450 in scup (Stegeman and Binder, 1979). Possibly, the fish used in our study had been exposed incidentally to aromatic hydrocarbons in the environment, and thus the extent of benzo(a)pyrene activation, as with the reported inhibition by 7,8-benzoflavone, may not be characteristic of an uninduced state.

A variety of primary and secondary metabolites of benzo(a)pyrene are formed by mammalian liver enzymes. Among the primary metabolites, benzo(a)pyrene-4,5-epoxide is the most potent mutagen, but this arene oxide is readily inactivated by epoxide hydrase, yielding benzo(a)-pyrene-4,5,-dihydrodiol (Levin et al., 1976). Benzo(a)pyrene-7,8-dihydrodiol is formed in a similar manner but, after further metabolism by cytochrome P-450, can yield benzo(a)pyrene-7,8-diol-9,10-epoxides--highly mutagenic secondary metabolites (Wislocki et al., 1976) that are not good substrates for, and thus not readily inactivated by, epoxide hydrase (Wood et al., 1976). An isomer of benzo(a)pyrene-7,8-diol-9,10-epoxide, more readily formed by 3-methylcholanthrene-induced cytochrome P-448 (Huberman et al., 1976; Yang et al., 1976), is believed to be the ultimate carcinogenic form of benzo(a)pyrene (Levin et al., 1977).

The patterns of metabolites of benzo(a)pyrene produced by scup and winter flounder have not been established. However, preliminary studies (Stegeman and Tjessem, unpublished) have indicated that little or no benzo(a)pyrene-4,5-dihydrodiol is formed in vitro by scup liver microsomes, whereas substantial amounts of 7,8-dihydrodiol and 9,10-dihydrodiol are formed. At the same time these studies confirm that epoxide hydrase is present in fish liver (Bend et al., 1977) and indicate that benzo(a)pyrene-4,5-epoxide is not responsible for mutation induced at least by the scup preparations. Further, formation of isomeric benzo(a)pyrene-7,8-diol-9,10-epoxide would be possible, although it is not known if any isomers of this metabolite would be preferentially formed.

The discrepancy between levels of benzo(a)pyrene hydroxylase activity and the activation of benzo(a)pyrene by the fish we studied indicates that metabolite patterns formed by these animals probably differ, although it is recognized that factors other than catalytic function of cytochrome P-450 may influence mutagenic activity detected using a PMS preparation (Ames et al., 1975). Yet the formation of mutagenic and carcinogenic diol-epoxides of polynuclear aromatic hydrocarbons by both species and sexes is a distinct possibility. It is known that patterns of metabolites formed by microsomes and intact cells can differ (Selkirk, 1977), but it is likely that toxic and mutagenic derivatives similar to those formed in vitro can result from metabolism in vivo in these and other (Ahokas et al., 1977) fish. Thus, the results clearly suggest that marine fish may be at risk to carcinogenic activity of polynuclear aromatic hydrocarbons known to be present in recent coastal marine sediments (Laflamme et al., 1978) and presumably coastal waters.

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HYPERSENSITIVITY FOR CARCINOGENESIS RESULTING FROM SPECIES HYBRIDIZATION: IMPAIRING CONTROL OF CELLULAR ONCOGENES AS TOOL TOWARDS TAILORING TEST ANIMALS SUITABLE FOR MONITORING CARCINOGENS

by

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ABSTRACT

Experimental strategies of selective breeding and mutagenesis/carcinogenesis have unveiled genes transmitted through the germ line in development of tumors of apparently non-viral etiology in the freshwater fish, Xiphophorus. It seems practicable to construct genotypes in which control of expression of cancer genes is impaired, although not abolished--a condition that renders them hypersensitive for carcinogens.

INTRODUCTION

The degree of genomic contributions to oncogenesis has been debated for some time. The most general genetic concept was proposed by Comings (1973), who postulated the existence of two classes of genes relevant to oncogenesis: the transforming gene (Tr) and the regulating gene (R) influencing expression of Tr. Oncogenesis in his model is thought to result from misguided expression of Tr, due to aberrant function of R. The recent molecular unveiling of cellular oncogenes in the genome of animal species (Bishop, 1978), mainly using retroviruses as experimental tools (Bishop, 1982; Varmus, 1982), and the finding of their likely involvement in oncogenesis in animals (Hayward *et al*., 1981; Payne *et al*., 1982), and possibly in humans (Cooper *et al*., 1980; Shih *et al*., 1981; Perucho *et al*., 1982), suggests that aberrant expression of cellular oncogenes transmitted through the germ line may at least be one cause of malignancy.

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Besides these recent molecular approaches to the role of what we call today oncogenes, the more classical experimental strategies of formal genetics have unveiled much earlier the existence of genes with the potential to elicit cancer ("cancer genes") in the genome of apparently normal organisms. Among others, hybrid tumors in plants [e.g., *Nicotiana* (Kostoff, 1930; *Datura* (Satina *et al*., 1950; *Sorghum* (Lin, 1969)] and in animals [e.g. carp (Sonstegard, 1977); duck (Crew and Koller, 1936); Sinclair swine (Millikan *et al*., 1974); xiphophorine fish (Gordon, 1959); *Drosophila* (Gateff, 1978)] as well as apparently genetic tumors in humans [e.g., neurobastoma (Knudson *et al*., 1971); Sparkes *et al*., 1979), polyposis of the colon (Lynch, 1967); melanoma (Anderson, 1971)] are most pertinent examples. Of these, the xiphophorine fish system may be regarded as one of the genetically best characterized models (for recent discussion, see Schwab, 1982a). With the knowledge that the presence of cellular genes elicits cancer in these fish, henceforth referred to as "oncogenes" or "cancer genes," our paper aims to evaluate the possibility of constructing hypersensitive genotypes particularly suitable for the detection of carcinogens in the water. We shall first give a brief overview on genetics of hybrid malignancy in the freshwater fish, *Xiphophorus*, review some of the results obtained with the primary carcingoen N-methyl-N-nitrosourea (MNU) as model agent, and eventually discuss some considerations for setting up a suitable test system.

GENES INVOLVED IN MALIGNANCY

Genes involved in malignancy were identified essentially by two experimental strategies: interspecies hybridization and carcinogenesis studies.

1. Interspecies hybridization

Gordon (1927), Kosswig (1927), and Haeussler (1927) observed after crossing procedures (Figure 1) that malignant pigment cell tumors ("melanoma") developed in backcross hybrids. Through subsequent studies today, at least four kinds of genetic loci involved in determining malignancy in *Xiphophorus* can be recognized and shall be subsequently discussed. It should be kept in mind, though, that their identification is soley based on formal genetics because nothing is known about their function, their products, and their architecture; the terminology used here is regarded only as operational. At least four loci contributing to the scenario of oncogenesis were identified: macromelanophore determinator (*Mel*), differentiated state (*Diff*), golden (*g*) and albino (*a*).

Mel

The simplest dermal pigment cell phenotype of wildtype *Xiphophorus* consists of melanophores, pterinophores, and guanophores distributed more or less uniformly. Within the melanophores, basically two types may be recognized: a small melanophore usually not exceeding 100 μm in diameter ("micromelanophore"), and a large giant melanophore usually approximating 1 mm ("macromelanophore"; Figure 2a). Little hard data exist on the

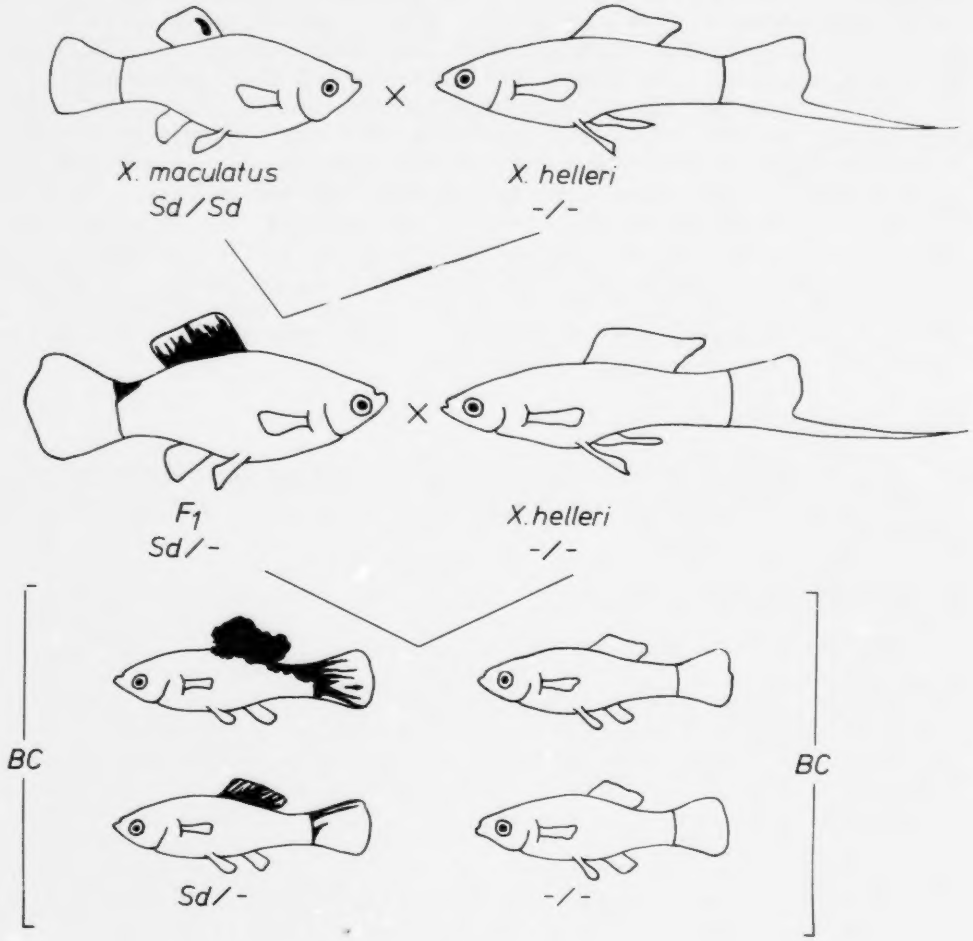
X. maculatus
Sd/Sd
X. helleri
-/-
F1
Sd/-
X.helleri
-/-
BC
BC
Sd/-
-/-

Figure 1. Experimental strategy of interspecies hybridization to identify cancer genes in the germ line; 25% of the backcross hybrids develop malignant melanoma resulting from introduction of the mel-locus Sd of X. maculatus into the genetic environment of X. helleri.

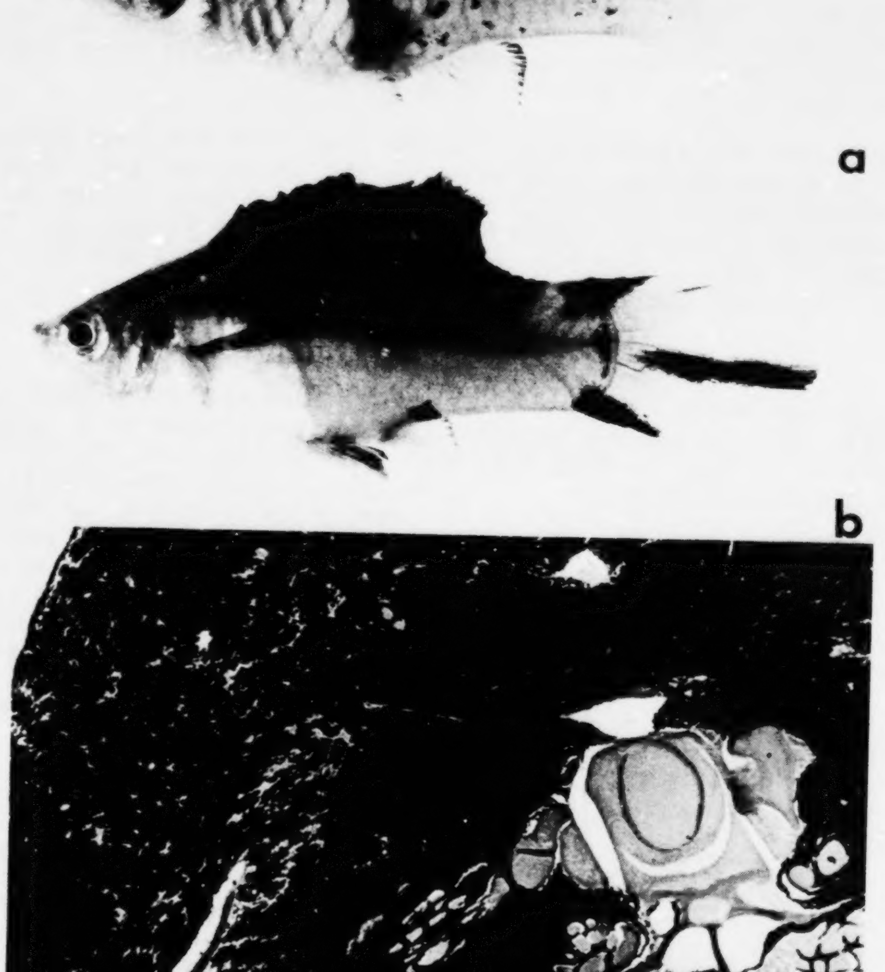

Figure 2. Phenotypes encoded by macromelanophore loci.
a. Colonies of macromelanophores determined by the loci Sd (spots in dorsal fin) and Sp (spots on body side).
b. Melanoma in BC-hybrid determined by mel-locus Sd.
c. Section through Sd-melanoma.

differentiation of the two melanophores and on their interrelation despite recent speculation (Anders *et al*., 1978). It seems established, though, that they are derived from the neural crest (Humm and Young, 1956). A general idea for their development would be that at some stage during differentiation two cell lineages are being determined of which one eventually yields micromelanophores, the other macromelanophores. The genetic determinator inducing macromelanophore development might actually be represented by what is referred to as *mel*.

Our report deals mainly with five *mel* -loci that have been described earlier in detail (Gordon, 1959; Anders *et al*., 1973a; Kallman, 1975): the X-chromosomal spotted dorsal (*Sd*) and spotted (*Sp*; Figure 2a) and the Y-linked stripe sided (*Sr*; Figure 6), all derived from *Xiphophorus maculatus*, and the X-linked lineatus (*Li*) and the Y-linked punctatus (*Pu*) derived from *X*. *variatus* (*not shown*).

Diff

Diff acts on the expressivity of *mel* and modulates the pigment cell phenotype. Genetic analyses unveiled this autosomal locus in *X*. *maculatus*. Homozygosity for *Diff* is associated with the development of terminally differentiated macromelanophores (Figure 2a). A single dose of *Diff* tends to inhibit the process of terminal differentiation leading to an increase of the ratio of incompletely versus terminally differentiated pigment cells. Lack of *Diff* causes the majority of the pigment cells to remain in an incompletely differentiated state, predominantly as melanocytes and melanoblasts, a condition that eventually leads to a massive increase in the number of pigment cells and to formation of a melanoma (Figure 2b,c).

Diff and *mel* are, as far as the loci *Sd* and *Sp* are concerned, not chromosomally linked. A marker gene esterase-1, coding for an electrophoretically identifiable product (Est-1), was identified to be chromosomally linked to *Diff*, thus allowing identification of the presence of *Diff* in any given genotype (Siciliano and Wright, 1976; Ahuja *et al*., 1980; Schwab and Scholl, 1981; Schwab, 1982b; Figure 3). For the *mel* -loci *Sr*, *Li*, and *Pu* a *Diff* -locus has not been identified. Crossing procedures that lead to melanoma in case of the *mel* -loci *Sd* and *Sp* generally fail to produce melanoma in case of *Sr*, *Li*, and *Pu*, except for rare cases, in which melanoma is produced in *Li* -genotypes. Mutagenesis may lead to a malignant phenotype, however, and it has been concluded that a *mel* -linked cis acting controlling element has been impaired by somatic mutation, releasing *mel* from negative control, perhaps exerted by a gene functionally homologous to *Diff* (Anders *et al*., 1973a,b). It may not be excluded, however, that positive control mechanisms also may operate in these cases, such as chromosome rearrangements leading to a configuration, in which *mel* is positioned in the vicinity of a transcriptionally active region ("insertion mutagenesis" in its broadest sense). The mechanism of *Diff* action, as that of *mel*, and their interaction in leading to the malignant phenotype, have not been defined. It may not be excluded that *Diff* represents a locus involved directly in differentiation of *mel* -determined macromelanophores, as hypothesized earlier (Vielkind, 1976), but it might also be envisioned that it could affect the pigment cell phenotype indirectly, e.g., by interfering with other genes conferring inhibition of pigment cell differentiation, or as a mutator gene. As it is, *Diff* seems to represent an important locus in regulating *mel* expressivity. Its property of not being linked to *mel* renders hybridization of the corresponding *mel* -carrying genotypes with others lacking *Diff* a powerful experimental tool of producing melanoma in a Mendelian fashion.

g

The effect of the autosomal recessive locus *g* can be best studied in *X. maculatus*. Animals homozygous for *g* virtually lack dermal melanophores, except for a few (Figure 4). A feasible hypothesis for the lack of melanophores is that differentiation of the dermal *X. maculatus* melanophores is blocked at an early stage, possibly at the stage of the chromatoblast, which is the common precursor for all dermal pigment cells. Guanophores and pterinophores are produced at normal level. Consequently, in *gg* individuals carrying *mel*, elimination of *Diff* fails to produce melanoma. Recent studies indicate that their inhibition of differentiation can be overcome by treatment with tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA; Schwab, 1982b), indicating their potential as test organisms for tumor promoting agents. Genetic studies have furthermore shown that macromelanophores determined by the various mel -loci differ: while *g* usually completely abolishes differentiation of macromelanophores determined by *Sd*, macromelanophores determined by other *mel* -loci, e.g., *Li*, differentiate to a normal extent, although micromelanophores are still lacking.

a

The autosomal recessive locus *a* is pleiotropic in the sense that it both confers the albinotic phenotype and exerts a stimulating effect on the degree of malignancy: pigment cells in albinotic melanomas show a lower degree of differentiation and a higher rate of division than in melanotic melanoma (Vielkind, 1976). Furthermore, carcinogen-induced neruoblastoma (Schwab *et al*., 1979) is considerably more malignant in albinotic than in wildtype animals. The mechanism for albinism is unclear, but *aa* -animals are tyrosinase positive (Vielkind, 1976; Schwab, 1982b).

2. Carcinogenesis studies

a. Hypersensitivity of interspecies hybrids

Studies with various carcinogens formerly used the majority of the non-hybrids of *Xiphophorus* available. No tumors were detected. However, recently, the nitrosamide N-methyl-N-nitrosourea (MNU) was employed in these studies for the following considerations. In general two types of chemical carcinogens can be recognized with respect to their mode of action: indirect acting and direct acting carcinogens. Indirect acting carcinogens, in order to become active, require metabolism by host enzymes for conversion to their active forms (Miller, 1970; Lijinsky, 1976; Bridges, 1976).

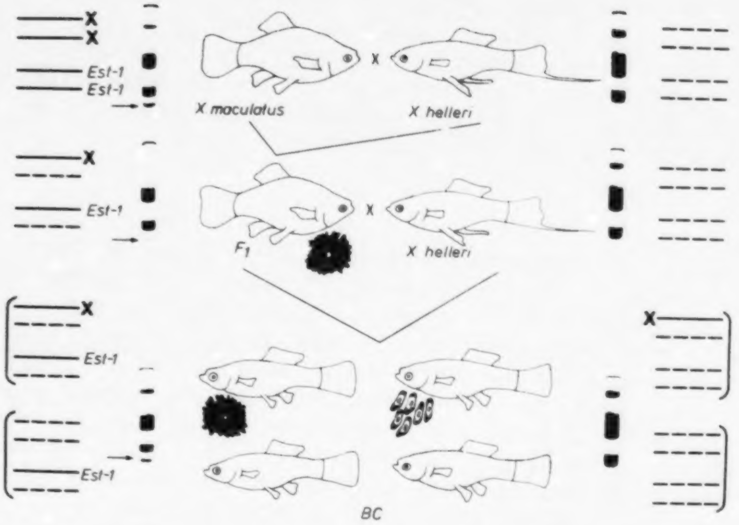
X
X
Est-1
Est-1
X maculatus
X helleri
X
Est-1
F1
X helleri
X
Est-1
Est-1
X
BC

Figure 3. Association of terminally differentiated macromelanophores with Est-1 marked chromosome of *X. maculatus*. Presence of Est-1 chromosome favors terminal differentiation, lack yields melanoma (according to Schwab and Scholl, 1980).

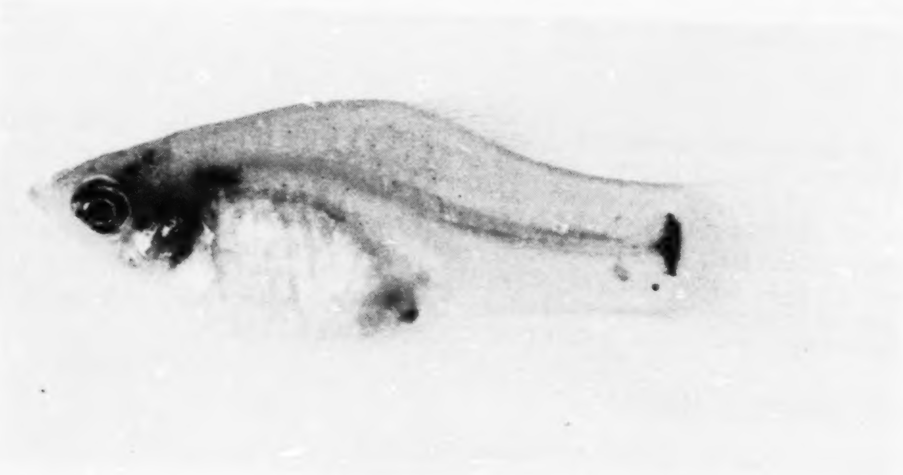

Figure 4. Effect of locus *g* on pigment cell phenotype. In *gg* animals carrying the *mel*-locus *Sd*, differentiation of dermal melanophores is blocked at an early stage. Few macromelanophores may develop, and retinal pigment cells are formed to normal extent. Melanoma does not occur in *gg* *BC*-hybrids due to differentiation block.

Consequently, in experiments using indirect acting carcinogens to analyze malignancy at least two genotype dependent variables must be taken into account: (1) enzymatic activation that may vary considerably in the different genotypes (Neubert, 1974), and (2) the susceptibility of the genotype to develop a tumor following reaction of the activated carcinogen with the target molecule in the cell. In addition, the enzymes involved in activation of the carcinogen may be present only in certain tissues and not in others due to differential gene activity, which may result in organotropic activity of the carcinogen. This kind of organotropy is due to organ-specific activation of the carcinogen, and not to the susceptibility of the particular genotype to respond to the ultimate carcinogen with development of cancer.

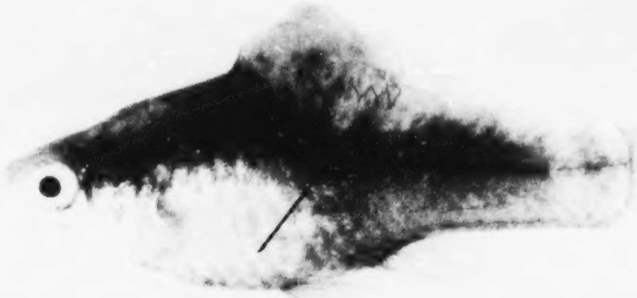

Figure 5. Effect of locus a on pigment cell phenotype. Pigment synthesis is abolished in aa animals, but amelanonic pigment cells develop. Degree of malignancy of melanoma in BC-hybrids is increased over that in wildtype animals.

In contrast, direct acting carcinogens undergo conversion to the ultimate carcinogen spontaneously. They may react instantly with the target molecules. Thus, by exposure to a direct acting carcinogen, the susceptibility of a genotype to a carcinogen should be tested directly. It is quite obvious that although indirect acting carcinogens are more common in the environment, direct acting carcinogens are more suitable for analyzing the genetic basis of susceptibility in an experimental system.

The nitrosamide N-methyl-N-nitrosourea (MNU) appears to be particularly suitable. First, MNU is a very potent direct acting carcinogen (Druckery *et .al*., 1965; Lijinsky, 1976; Narisawa *et al*., 1976). Second, MNU data suggest that mutation is the primary event that induces transformation of normal into malignant cells (Bouck and Mayorca, 1976). Third, MNU penetrates all tissues of the animal (Magee *et al*., 1975).

The recent carcinogenesis study was extended beyond non-hybrids to F1 and backcross hybrids (BC) that have a spontaneous rate of tumor incidence below level of detection. The experimental strategy, aiming to identify chromosomes conferring hypersensitivity, was based on the following considerations.

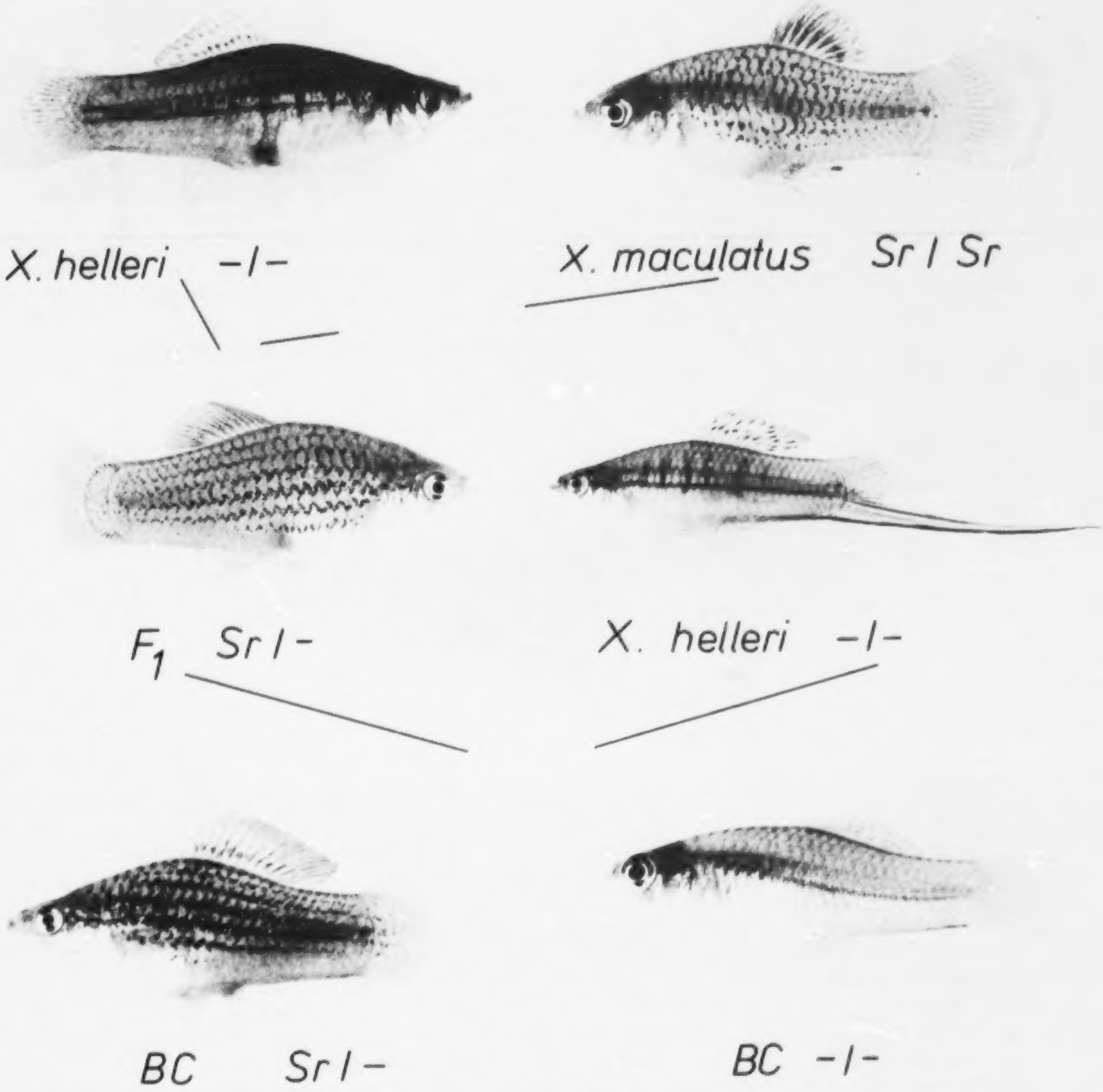

Figure 6. Experimental strategy of combined selective breeding and mutagenesis/carcinogenesis to identify cancer genes in the germ line. A genetically defined chromosome (in this case the Y-chromosome marked by the mel-locus Sr) of one species (X. maculatus) is introduced by selective breeding into the genetic milieu of another species (X. helleri). BC-segregants differ with their gene pool only by the Sr-chromosome. Segregants are treated with mutagens/carcinogens and their susceptibility is compared. The Sr-chromosome in this case confers hypersensitivity for melanoma, -/- segregants are largely resistant.

The various species of *Xiphophorus* are phenotypically characterized by the presence of spot patterns consisting of melanophores, guanophores, and pterinophores. The corresponding pigment cell loci are expressed codominantly, and the patterns can be used therefore for identifying chromosomes in any hybrid genotype and, furthermore, as convenient phenotypic markers for selective breedings (for details on the spot patterns see Gordon, 1959; Anders *et al*., 1973a,b; Kallman, 1975). The basic strategy, displayed in the crossing scheme in Figure 6, was to introduce defined chromosomes from one species into the genetic environment of another by crossing and backcrossing. The *BC* segregates with respect to the marker chromosome, and the gene pool of the *BC*-segregants differs only with respect to this chromosome (for higher *BC*-generations the statistics is better than for lower ones).

The result of the previous study confirmed that nonhybrids proved to be completely resistant (Schwab and Anders, 1981). In contrast, *F1* hybrids showed slight sensitivity and developed melanoma (0.6% incidence), while backcross hybrids responded to MNU-treatment with the development of a large spectrum of tumors, including melanoma, neuroblastoma, carcinomas, fibrosarcoma, rhabdomyosarcoma, and lymphosarcoma (Schwab and Anders, 1981). By far most of the tumors were represented by melanoma (in 254 of about 5100 fish treated; Figure 7), followed by neuroblastoma (66 cases; Figure 8), fibrosarcoma (50 cases; Figure 9), and carcinoma (12 cases; Figure 10). Other tumors, as the rhabdomyosarcoma (Figure 11) were rare (1 safe case).

Figure 7. Melanoma induced by MNU.

Sensitivity in the *BC*-hybrids was not distributed at random. Instead, certain hybrids showed hypersensitivity for certain tumors with incidence up to 15% under the experimental conditions. Appearence of some tumors could be assigned to genetically defined chromosomes, particularly the neuroblastoma in hybrids derived from *X. variatus* - *helleri* associated with the *Li* -chromosome of *X. variatus* (Schwab *et al*., 1979), while the fibrosarcoma in the same hybrids seems to be associated with an autosome (Schwab *et al*., 1978). Melanoma in hybrids derived for *X. maculatus* - *helleri* is associated predominantly with the *mel* -locus *Sr*, and the X-chromosome defined by alleged deletion of *Sd* (Schwab and Scholl, 1981).

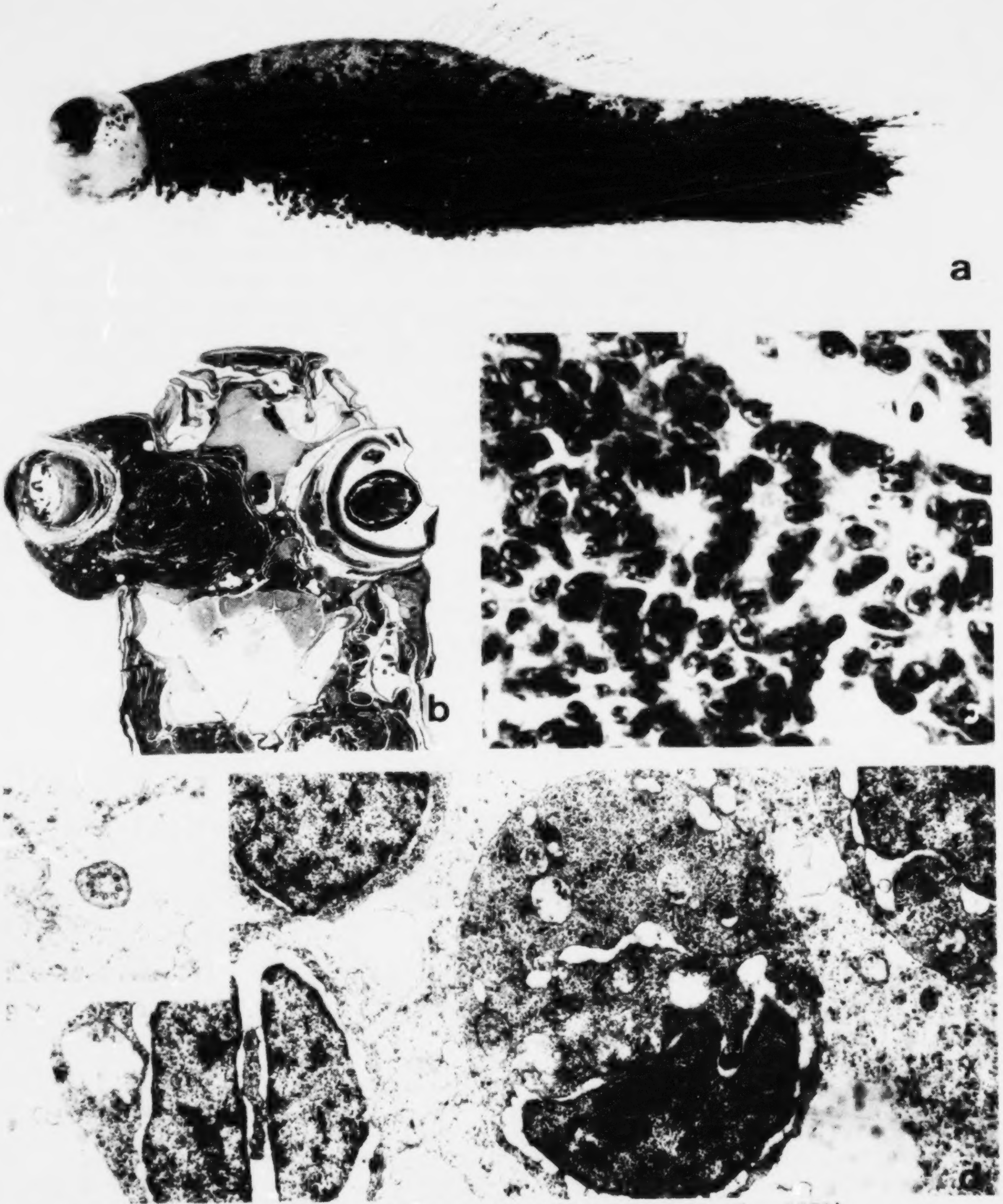

Figure 8. Neuroblastoma induced by MNU (from Schwab *et al*., 1979). a. Fish; b,c. Section; d. EM-section. Insert shows typical cilium with 9=0 pattern of microtubles.

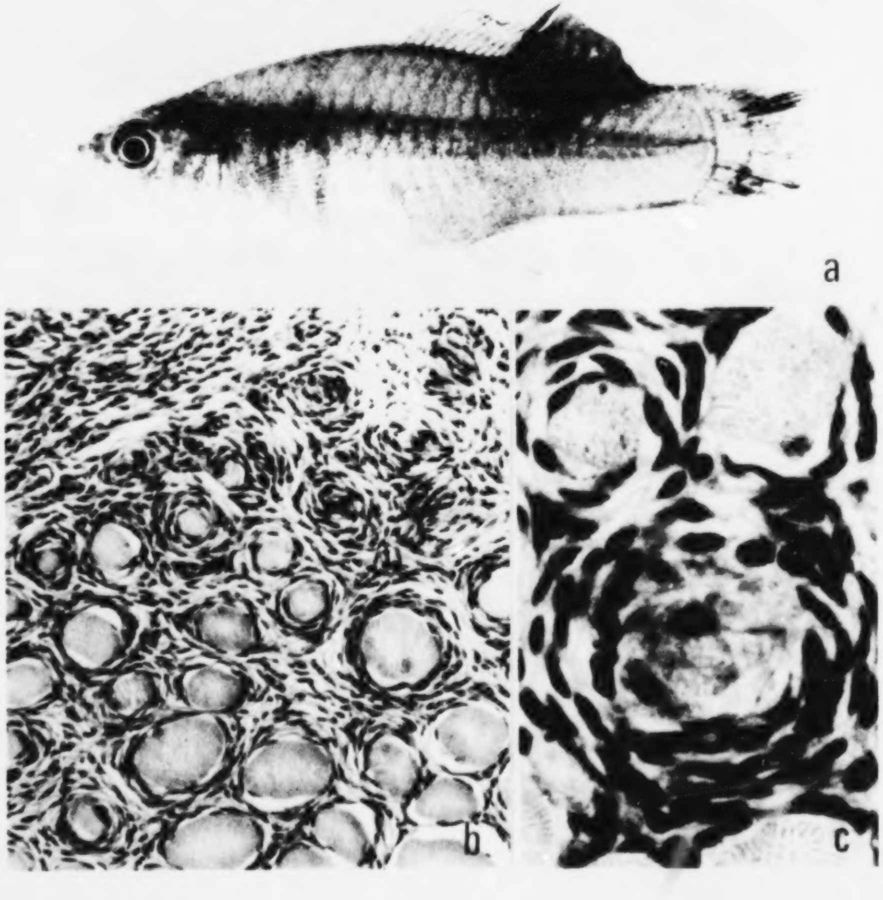

Figure 9. Fibrosarcoma induced by MNU (from Schwab et al., 1978).
a. Fish
b. Section showing invasion into muscle tissue.

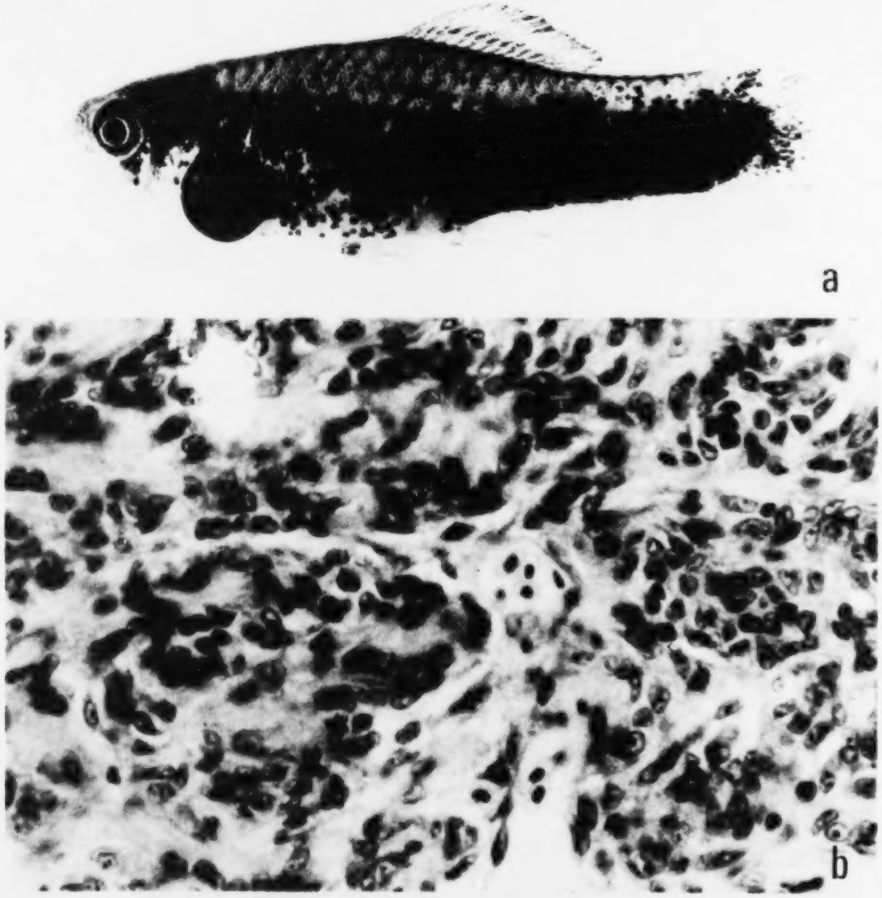

Figure 10. Carcinoma induced by MNU.
a. Fish
b. Section

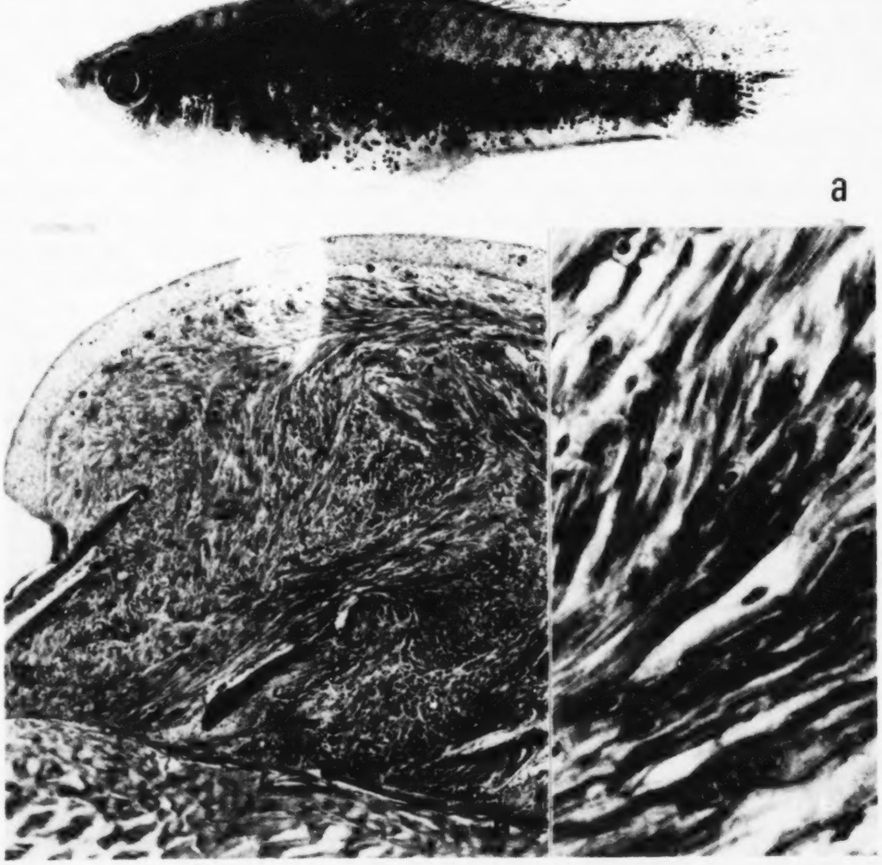

Figure 11. Rhabdomyosarcoma induced by MNU (from Schwab _et_ _al_., 1978).
a. Fish
b,c. Section

In summary the results of our study indicate that interspecies hybridization obviously leads to impairment of control of cellular transformation, a condition that confers sensitivity for induction of malignancy by carcinogens. Hypersensitivity is associated with genetically defined marker chromosomes.

b. Genetic modification of MNU-induced melanoma

As in spontaneous hybrid melanoma, two phenotypes could be recognized in the MNU-induced melanoma: one benign and the other malignant (Schwab and Scholl, 1981). We attempted to examine whether the degree of was malignancy associated with Diff. An easy handle for testing this possibility was to monitor for the Diff -linked Est-1. We found that an association between the benign phenotype and presence of Est-1 and the malignant phenotype and absence of Est-1 could be established (as in Figure 3), thus making an involvement of Diff in the control of the MNU-induced pigment cell phenotype likely (Schwab and Scholl, 1981; Schwab, 1982b).

SIGNIFICANCE - POSSIBILITIES - POTENTIALS

Experimental carcinogenesis in small aquarium fishes has been carried out with several species and a variety of chemical carcinogens (Matsushima and Sugimura, 1976). In recent years, it has been recognized that small aquarium fishes offer some advantages for carcinogenesis studies: mainly, fish are more sensitive to carcinogens and, at the same time, are more resistant to toxic effects than are rodents (Matsushima and Sugimura, 1976). In addition, large numbers of animals can be raised and maintained easily. Our investigation revealed that a large variety of tumors can be induced by treatment with MNU in *Xiphophorus*. Most have been observed to develop spontaneously in fishes (Ashley *et al*., 1979; Mawdesley-Thomas, 1975; Harshbarger, 1973). However, they apparently have not been observed thus far in tests with carcinogens (Matsushima and Sugimura, 1976).

Species differences in the sensitivity to respond with tumor development to carcinogen treatment have been reported. For instance, different inbred strains of mice were found to vary in their response to skin carcinogenesis (Berenblum, 1974). Boxer dogs are particularly susceptible to tumors following treatment with MNU (Denlinger *et al*., 1978) and show also a much higher spontaneous tumor incidence than other breeds (Cohen *et al*., 1974). Further, in experiments with the guppy (*Lebistes reticulatus*), hepatic tumors were induced by nitrosamines (Sato *et al*., 1973; Pliss and Khudoley, 1975), whereas Scherf (1976) showed that the same species, although most likely another strain, was resistant. All these results, including our investigation, clearly show the involvement of genetic factors in susceptibility and urge the use of highly defined genotypes in carcinogenesis studies.

Differences in susceptibility are often attributed to variations in the rate of generation of the ultimate carcinogen. For our study this explanation is rather unlikely: several genotypes found to be sensitive or resistant to MNU proved to be also sensitive or resistant to X-rays, particularly in the case of the melanoma and the neuroblastoma (Schwab *et al*., 1979; Schwab and Anders, 1981). Such parallels in the sensitivity spectrum make it likely that, in short, genetic factors operate directly in determining sensitivity and resistance.

The fact that hypersensitive genotypes can be constructed from genotypes resistant to cancer by selective matings gives further support to the idea that one or several genetic changes are involved in the etiology of cancer. In the present case of hypersensitivity, apparently new combination of chromosomes, as well as possibly mutational events, could result in, using the terminology of modern molecular genetics, enhancement of expression of a gene with the potential of triggering malignancy. Basically two molecular mechanisms could be envisioned. One is impairment of negative control as proposed earlier (Schwab *et al*., 1979; Schwab and Anders, 1981); the other is chromosomal rearrangement setting up a positive control mechanism, e.g. insertion mutagenesis in its broadest sense, resulting in placement of the corresponding oncogene upstream or downstream in the vicinity of a promoter sequence, as found in chickens using retroviruses as mutagens (Varmus, 1982; Bishop, 1982).

The molecular nature of the oncogenes identified in *Xiphophorus* by genetic strategies remains to be elucidated. It is unlikely from the present data that any one of the homologues of retrovirus transforming genes identified by molecular studies in the germ line of *Xiphophorus* (manuscript in preparation) is involved in oncogenesis. As it is, construction of genotypes by selective breeding carrying several oncogenes conferring hypersensitivity should be a powerful tool for creating suitable test organisms and at the same time may provide further clues towards an understanding of cancer genes in malignancy.

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THE USE OF GENETICALLY MODIFIED FISH IN THE DETECTION AND MEASUREMENT OF CARCINOGENS IN WATER

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ABSTRACT

The platyfish, *Xiphophorus maculatus*, was challenged with small concentrations of four carcinogenic polycyclic hydrocarbons. Using the ratio of melanized fin area to total fin area, we observed that the fin spot increased in direct proportion to dosage and duration of exposure. Our tests demonstrate that the response, which involved increased cellular activity, can be used as an *in vivo* monitoring system for the presence of carcinogens in water.

INTRODUCTION

As carcinogenic substances become more common in the environment, the need for a sentinel biosystem capable of monitoring chemicals in our waters becomes more urgent. It is important to search for a *in vivo* system that can rapidly and inexpensively test not only a single compound, but the entire enviromental load of contaminants.

A system has been developed for airborne pollutants by using *Tradescantia puldise* and color mutations of the flowering parts (Sparrow *et al*., 1974). This assay monitors the total environment rapidly and inexpensively. A bioassay with similar advantages is greatly needed to monitor the aquatic environment.

Stich and Acton (1976), in a study of "early warning" systems, found the flatfish, *Pleuromectes sp*., to be a promising research animal for carcinogen detection. A great deal of the work involving the relationship between neoplasias in fish and pollutants has used the commercially significant flatfish (Mearns and Sherwood, 1976; Bucke, 1976), which, however, are neither genetically controlled nor readily adaptable to laboratory conditions.

Tumor induction has been studied in guppies and zebra fish (Pliss and Khudoley, 1975), and in the medaka, Oryzias latipes, (Ishikawa et al., 1975) by organic carcinogens. Matsushima and Sugimura (1976) have reported experimental carcinogenesis in small aquarium fish. These experiments involved liver tumors which, unfortunately, cannot be examined in the living fish.

Ames (Ames et al., 1973) has developed an excellent experimental design that employs revertant frequency analysis to draw a correlation between known carcinogens and the production of mutations in Salmonella typhimurium. This rapid and convenient bioassay has gained wide acceptance in testing laboratories and bids well to become accepted an criterion of mutagenesis. However, mutagenesis and carcinogenesis are not always synonymous. Therefore, an additional system is needed to measure carcinogenecity.

In their review, Stich et al. (1975) have stressed the necessity for a practical screening assay that would actually involve a carcinogenic process, i.e., morphological transformation. Such an assay, ideally, would involve a vertebrate with a thoroughly known genetic background, preferably isogenic.

The most commonly used test animal for detection and dosage studies involving carcinogens is the mouse. However, the use of small mammals in laboratory tests is not only time-consuming, but also enormously expensive. It would be preferable to have a backup sentinel system in which a reasonably short exposure to the suspected carcinogen results in a visible, or measurable, cellular change that could be recognized as atypical growth. One such animal might be the small poeciliid fish, Xiphophorus maculatus [the platyfish (P^{sd})]. The platyfish possesses a melanized spot on the dorsal fin which, in and of itself, is not particularly significant. However, it can be, and has been, genetically altered in various ways and therefore is particularly suitable for detection of carcinogens dissolved in water.

One mechanism of alteration involved crossing Xiphophorus maculatus to a closely related species, Xiphophorus helleri [the swordtail (S)]. The resultant hybrid (P^{sd}xS) exhibited a dorsal fin melanophore hyperplasia known to be pretumorous. Experiments of Gordon (1951), Anders (1973), and Kallman (1973) have demonstrated that the production of atypical growths in the poeciliid fish involved the derepressive action of the swordtail genome upon genes occurring on the X chromosome of the platyfish. As a result of their research, it is known that the susceptibility of the hybrid to tumor production may be increased or decreased, almost at will. When the hybrid is backcrossed to the swordtail, fish developed with dorsal fin pigment cells which become true malignant melanomas. On the other hand, it was possible to prepare crosses in which the fish were genetically poised on the brink of tumor production and a small chemical "push" would be sufficient to start the process of overgrowth and loss of contact inhibition, the first detectable step toward malignancy. This can be achieved by backcrossing the hybrid to a platyfish.

Platyfish pigment cells also can be altered by X-irradiation to obtain a viable method of testing carcinogens in water using fish with impaired control of pigment cell production. Such a group, the sd'sr' platyfish, was developed by Dr. Fritz Anders of the Genetisches Institute der Justus-Liebis-Universitat, Geisen, West Germany. These fish differed from their predecessors in that the gene for striped-side was translocated from the X chromosome and become attached to the Y chromosome. This translocation is associated with a loss of repressor activity which appears to be a significant factor in the sensitivity of dorsal fin melanophores to carcinogens (Anders, personal communication).

TEST METHODS AND MATERIALS

Experiments performed in our laboratory were designed to test both groups of fish for their susceptibility to carcinogen-induced melanoma production and to determine which fish would provide the most useful organism for future carcinogenic testing. The stocks of sd'sr' platyfish in our experiments were an inbred stock generously provided by Dr. Fritz Anders. They were subdivided into male and female groups because the involvement of the X and Y chromosomes in the pigmentation coding indicated that response of the sexes would differ.

Platyfish with spotted dorsal fins (P^{sd}), *Xiphophorus maculatus*, and green swordtails (S), *Xiphophorus helleri*, were obtained from the Genetics Laboratory of the New York Zoological Society. The swordtail females were artificially inseminated with the sperm of the platyfish males. The hybrids, being sexually compatible with the parent species, were easily mated to platyfish. The offspring are predominatly female; only females were used.

All fish were raised to the age of 6 to 8 months and then sexed, weighed, and photographed. Benzo(a)pyrene (BaP), 3,4,9,10-dibenzpyrene (DBP), 3-methylcholanthrene (MC), and benzanthracene (BA) were dissolved independently in 600 mℓ of aged tank water. Fish were exposed individually to a single carcinogen for 4 days, and then transferred to fresh water and fed brine shrimp.

The carcinogens (concentration, 25 μg/mℓ water/gm of fish) were suspended in 2 mℓ of Carbowax 200 (Atlas Powder Co.) as an inert vehicle. The test compounded BaP, DBP, MC, and BA were obtained from Sigma Chemical Co. and used without further purification.

Each group was controlled by a fish of its own type and comparable spot size. The control fish were kept in aged tank water which contained 2 mℓ Carbowax 200, but no known carcinogens. Additionally, a group of similar fish were exposed to chrysene as a noncarcinogen with the same overall chemical structure as the carcinogens. There was no measurable increase or growth in the spot area of the control of chrysene-treated fish. During other experiments, diphenylnitrosamine was added as a noncarcinogenic compound. However, its toxicity was so great that its use had to be discontinued.

Photographs of the dorsal fin were taken every few days before and after exposure to carcinogen for 1½ months. A 55-mm macro-lens was used for all pictures. The fins and spots were measured by a planimeter, and the data recorded as a ratio of the area of the fin covered by melanized spot vs. the total fin area.

RESULTS

The measurements of spot vs. fin area from the three experiments are collated in Tables 1, 2, and 3. The omissions in the tables are due to the difficulty in taking pictures of live fish. The fish had to be correctly positioned, the fin perfectly erect, and the melanophores completely expanded. Many of our photographs were unusable for various reasons. Much of the scatter seen in our data is due to such complications.

Figures 1, 2, 3, and 4 represent the growth of the pigmented spots based on the most reliable data. The slopes of the lines drawn determined by linear regression analysis are presented at the bottom of the columns on the corresponding table. Figures 1, 2, and 3 indicate that the response of the three types of fish varied widely. The response of the various fish to methylcholanthrene is shown in Figure 1.

The response of the $p^{sd'sr'}$ male was very marked when compared with the control. The $P^{sd'sr'}$ female, and the $(P^{sd}xS)p^{sd}$ changed only slightly as compared with the control. The phenomenon was repeated (Figures 2 and 3) with both benzo(a)pyrene and 3,4,9,10-dibenzpyre In each case, the response of the $P^{sd'sr'}$ males was more rapid than the other two. Often, the $P^{sd'sr'}$ females and the $(P^{sd}xS)$ P^{sd} females did not respond at all; spontaneous pigment cell spot growth was observed in the $(P^{sd}xS)P^{sd}$ controls which rendered them less valuable as a comparison standard. In contrast, the $P^{sd'sr'}$ males gave a marked response to all the polycyclic hydrocarbons used, demonstrating that the $P^{sd'sr'}$ male was the most useful for the testing of carcinogens in aqueous solutions.

CONCLUSIONS

We believe that there is a real need for a rapid detection system for carcinogens as distinct from mutagens. This system should employ an eucariot and, preferably, a vertebrate. Tests for mutagenicity on bacteria are useful screening devices, but shoud not be considered synonymous with tests for carcinogenicity. We agree that a single mutation is probably not sufficient to produce carcinogenesis in higher organisms.

Therefore, we feel that a test system using genetic manipulation to produce an organism which is already "primed" genetically has a very good chance of satisfying the need for a rapid and relatively inexpensive test system for carcinogens. To date, our preliminary data indicate that the pigment cells under study exhibit at least one criterion of atypical growth--namely, loss of contact inhibition.

TABLE 1. RESPONSE OF THE SWORDTAIL-PLATYFISH HYBRID BACKCROSS $[(P^{sd}XS)P^{sd}]$ TO POLYCYCLIC HYDROCARBONS IN WATER

	Exposure Substance							
DAY	CW[a]	CW	MC[b]	BaP	BaP	DBP	BA	BA[c]
3	-	-	-	19.11[d]	-	-[f]	25.92	12.30
4	22.76	-	-	20.65	-	-	23.91	-
7	-	-	11.32	-	23.97	16.42	18.09	8.77
10	-	20.25	-	-	27.57	-	23.28	9.63
12	30.24	24.13[e]	13.73	19.19	29.21	-	26.69	18.95
13	35.80	21.87	-	-	-	21.18	24.72	12.35
17	36.15	-	13.82	25.03	29.94	-	-	18.12
18	-	22.12	-	-	-	22.88	23.12	17.81
20	-	-	13.54	-	-	-	27.54	18.13
21	-	-	-	-	-	24.99	-	-
25	38.78	26.29	14.65	-	-	26.09	25.61	19.80
27	-	-	-	-	-	23.20	29.50	-
$\sqrt{r^2}$	.912	.776	.853	.715	.896	.868	.560	.701
m	.772	.301	.152	.298	.567	.408	.227	.435

a. Control fish (CW) exposed to 0.3% Carbowax 200 in acclimatized tank water.

b. The carcinogens, 3-Methylcholanthrene (MC), Benzo(a)pyrene (BaP), 3,4,9,10-Dibenzpyrene (DBP), and 1,2-Benzanthracene (BA) suspended in Carbowax 200 to a final concentration of 25 μg/mℓ of water/gm of fish; duration of exposure to carcinogens: 4 days at 23° C.

c. Each column represents the data obtained from a single fish.

d. Data expressed as percent of total dorsal fin area covered by pigment cells.

e. Some daily variation in measurements is encountered due to the fact that it was not always possible to photograph the fin in a fully extended position.

f. Not measured.

TABLE 2. RESPONSE OF THE FEMALE SD'SR' PLATYFISH ($P^{sd'sr'}$) TO POLYCYCLIC HYDROCARBONS IN WATER

	Exposure Substance						
DAY	CW	MC	MC	BaP	BaP	DBP	BA
1	-	-	-	28.10	-	-	-
10	9.60	19.30	57.00	28.30	6.99	-	19.95
15	-	-	58.20	27.00	8.00	-	26.70
22	9.00	24.60	60.80	-	8.60	8.90	-
24	8.50	21.60	58.40	-	-	12.10	25.80
29	-	-	59.60	-	8.50	14.60	26.20
38	8.40	-	-	-	10.30	12.90	-
43	9.30	-	69.40	-	10.40	-	-
$\sqrt{r^2}$	.384	.744	.896	.675	.964	.580	.679
m	-.015	.261	.351	-.067	.099	.194	.250

TABLE 3. RESPONSE OF THE MALE SD'SR' PLATYFISH ($P^{sd'sr'}$) TO POLYCYCLIC HYDROCARBONS

	Exposure Substance					
DAY	CW	MC	MC	BaP	DBP	BA
1	38.90	25.80	-	20.10	-	-
6	32.70	-	18.70	-	-	-
7	-	23.60	22.30	20.70	25.80	27.40
9	31.20	29.20	20.60	22.50	33.80	-
13	-	29.30	19.40	23.80	37.00	22.30
14	34.30	-	-	-	36.80	25.20
24	-	38.10	24.90	-	-	26.60
$\sqrt{r^2}$	.590	.911	.850	.932	.904	.024
m	-.360	.588	.265	.315	1.430	.008

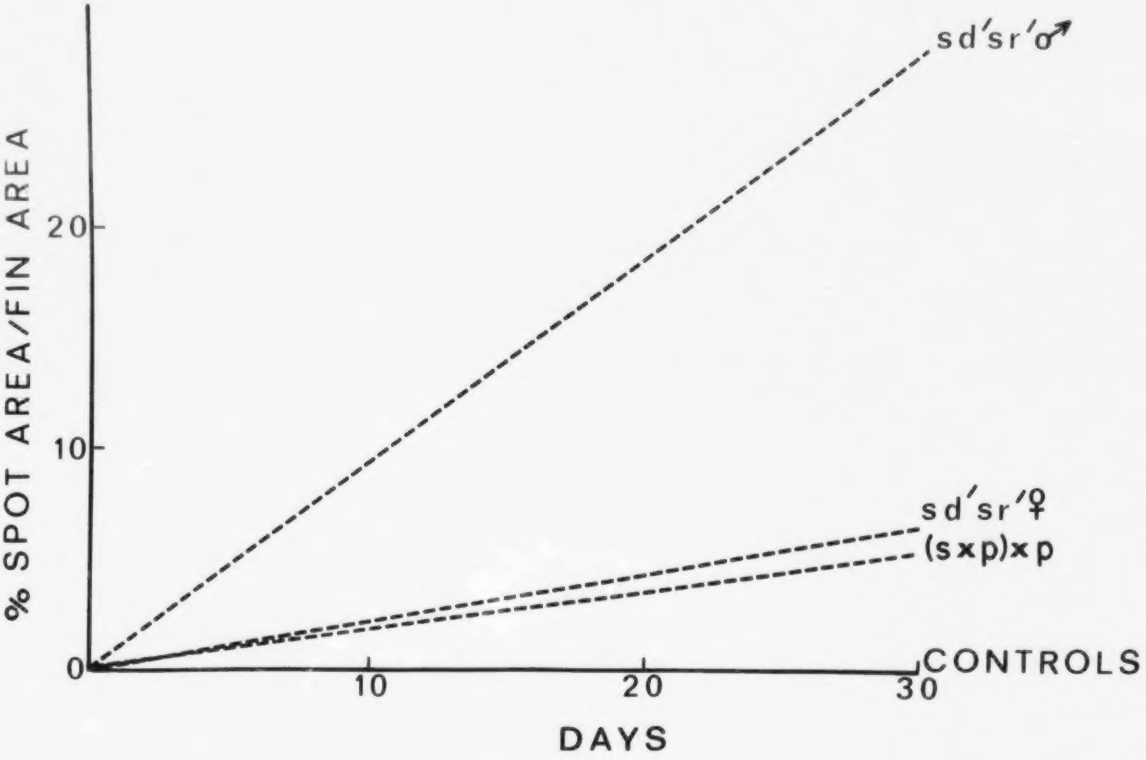

Figure 1. The difference is shown in the response to methylcholanthrene of three fish. It is derived from data in Tables 1, 2, and 3 by setting the appropriate controls as 0 growth and plotting the rate as (spot area/fin area X 100) against time. The equation for the lines drawn were determined by subtracting the slopes of the controls from slopes of the experimentals.
$m_{final} = m_{exper.} - m_{control}$

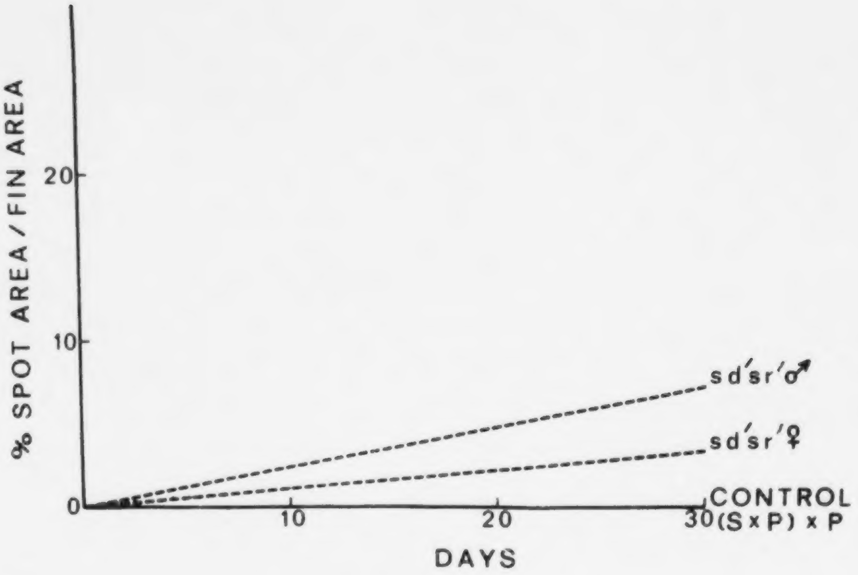

Figure 2. Response of the three fish to benzo(a)pyrene derived in the same manner as Figure 1.

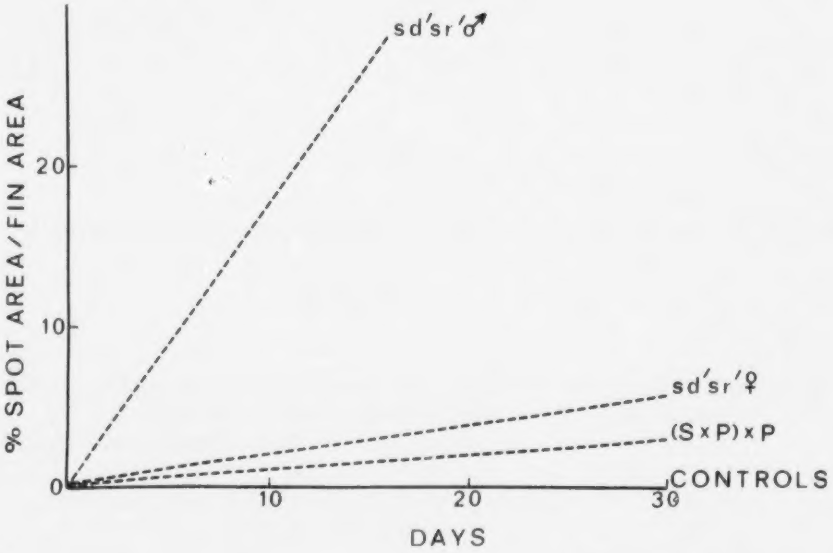

Figure 3. Response of the three fish to 3,4,9,10-dibenzpyrene derived in the same manner as Figure 1.

The possibility exists that these genetically metastable cells have responded by growth and not by malignant transformation. An increased time of exposure to the hydrocarbons might well result in the transformation of the pigment cell, in addition to the stimulation we have reported.

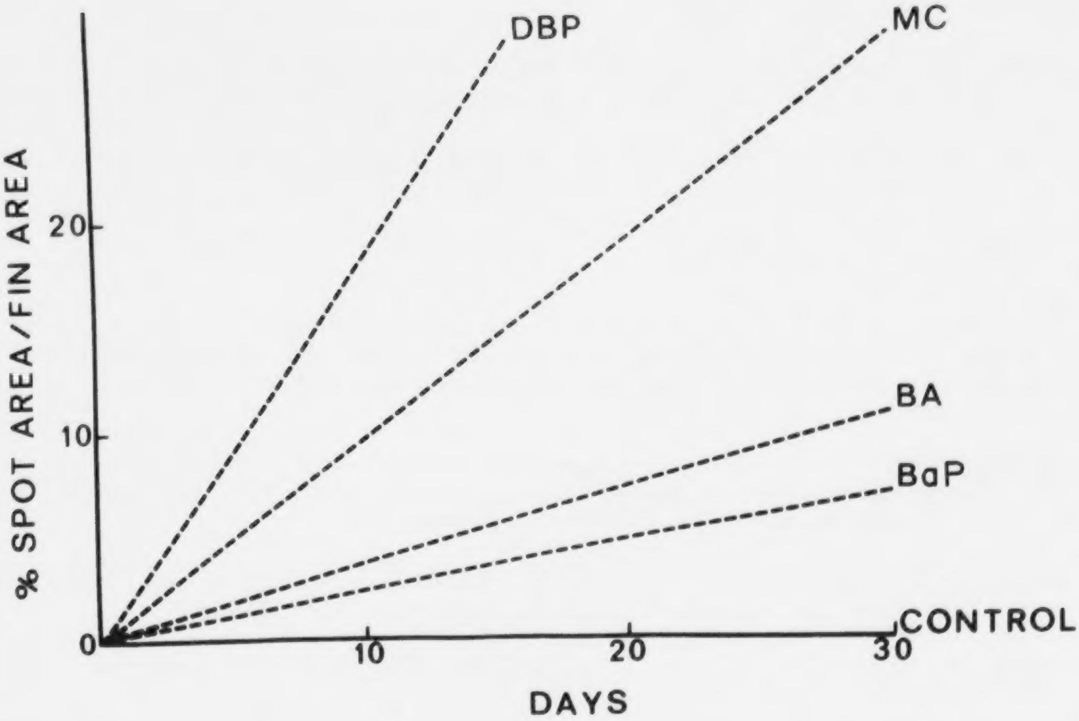

Figure 4. Graph showing the response of the sd'sr'♂ platyfish to the 4 polycyclic hydrocarbons. According to our data, MC and DBP are are more effective in eliciting a response in the platyfish pigment cells.

ACKNOWLEDGMENTS

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THE DISTRIBUTION OF BENZO(a)PYRENE IN BOTTOM SEDIMENTS AND OF NEOPLASMS IN BOTTOM-DWELLING FLATFISH SPECIES OF THE PACIFIC AND ATLANTIC OCEANS, NORTH, CHINA, BERING AND BEAUFORT SEAS, AND SEA OF OKHOTSK

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ABSTRACT

Human and animal populations are continuously exposed to hundreds of carcinogenic, mutagenic, clastogenic and recombinogenic agents. These chemicals can interact, leading to extremely large numbers of possible permutations and combinations that can either enhance or reduce their genotoxic or carcinogenic activity. It is impossible to examine all these interactions for economic and logistic reasons. Thus, other approaches to identify high risk areas must be sought. In this paper, we explore the feasibility of using naturally occurring animal populations as an early warning system to detect carcinogen-mutagen contamination of a particular environment. The basic idea is simple. In animals, tumors may appear within months, whereas in man the latency period may well exceed 16 or more years. Thus, by screening indigenous animal populations for benign and malignant neoplasms, we should be able to recognize carcinogen-contaminated areas long before they adversely affect man. There is no shortage of examples of making use of "built-in" organisms to detect agents with a general toxic action. However, the practicability of a naturally occurring indicator organism for chemical carcinogens or mutagens is still unproven. In this paper, we critically review the difficulties encountered in the attempt to use neoplasms of bottom-dwelling fish populations as a possible indicator for man-made or naturally ocurring contamination of shallow estuarine nursery grounds that might at some time be developed for intensive aquacultural usage.

INTRODUCTION

Epidemiological studies of human populations coupled with chemical analysis have made it possible to identify numerous carcinogenic and mutagenic agents in man's environment and to trace their origins. Based on this experience, it has been proposed that naturally occurring animal populations be used in an early warning system to detect carcinogen-mutagen contamination of a particular environment. The basic idea is simple. In animals, tumors may appear within months, whereas in man the latency period may well exceed 16 or more years. Thus, by screening indigenous animal populations for benign and malignant neoplasms, we should be able to recognize carcinogen-contaminated areas long before they adversely affect man. There is no shortage of examples of making use of "built-in" organisms to detect agents with a general toxic action. However, the practicability of a naturally occurring indicator organism for chemical carcinogens or mutagens is still unproven. In this paper, we critically review the difficulties encountered in the attempt to use neoplasms of bottom-dwelling fish populations as a possible indicator for man-made or naturally occurring contamination of shallow estuarine nursery grounds that might at some time be developed for intensive aquacultural usage.

Advantages of Flatfish as an Indicator Organism

In choosing a suitable animal test species, we applied several criteria (Stich and Acton, 1976; Stich et al., 1976a,b): (1) Availability. Flatfish samples can be easily collected by bottom trawling in shallow waters, requiring only a small boat and a trawl net (available at all marine stations). Moreover, several flatfish species are of commercial importance and thus can be obtained on regular fishing boats or at processing plants. (2) Global distribution. Flatfish have also the advantage of a wide geographic distribution. Some species are common along the shores of the entire northern Pacific Ocean or northern Atlantic Ocean. (3) Migratory behavior. Several flatfish populations have nursery grounds in the shallow waters of estuaries or within deltas of rivers that are areas of greatest concern because of their potential for becoming polluted. The movements of juvenile populations into deeper waters and their migratory pattern as adults are fairly well-known. (4) Tumor prevalence. Papillomas have been reported to occur among flatfish at such high frequencies that their precise quantitation would require the screening of relatively only small numbers (Nigrelli et al., 1965; Wellings et al., 1965, 1977; Cooper and Keller, 1969; Wellings, 1969; Stich et al., 1976b, 1977a; Yamazaki et al., 1978). (5) Age-adjusted tumor prevalence. Skin papillomas develop on young flatfish. Skin nodules which precede papillomas start to appear on lemon soles, *Parophrys vetulus*, about 4 to 5 months after metamorphosis and reach a peak within the first year post-metamorphosis. A valid comparison can only be achieved by comparing the peak frequencies at each sampling station. (6) Diagnosis. The skin papillomas are easily detectable by macroscopic examination. At the microscopic level, papillomas are characterized by rounded enlarged cells with vacualization and degeneration of cytoplasmic organelles (Wellings et al., 1965, 1977; Brooks et al., 1969; Wellings, 1969; Stich et al., 1976b, 1977a; Yamazaki et al., 1978).

It was suggested that these cytoplasmic organelles, the so-called "X" cells (Brooks *et al*., 1969) could be protozoa (Wellings *et al*., 1977), a hypothesis which we have previously criticized and which seems to lack factual support (Peters *et al*., 1978).

Restriction of the Flatfish-Tumor System: Geographic Distribution

If the flatfish-papilloma system has the many advantages mentioned above, the questions must be asked, why then do we not have a "fish tumor watch", or why have regulatory agencies not yet introduced a fish-tumor monitoring program in areas of aquaculture and recreational activity. One of the reasons is the unique global distribution pattern of the skin papillomas (Figure 1). Flatfish with skin nodules and papillomas characterized by typical X-cells and envelope cells seem to be restricted to the northern Pacific Ocean and the adjacent Bering Sea and Sea of Okhotsk. To the best of our knowledge, papillomas composed of X-cells have not been seen outside of what we previously called areas of potential skin papilloma risk (Stich *et al*., 1977a). Of four skin papillomas of flatfish, *Platichthys flesus*, caught in the North Sea, all lacked the X-cells and their histopathology was more comparable to the skin papillomas found among European eels. Obviously, any correlation between tumor frequency and level of carcinogens in the marine environment can be sought only in those regions in which flatfish can develop skin papillomas.

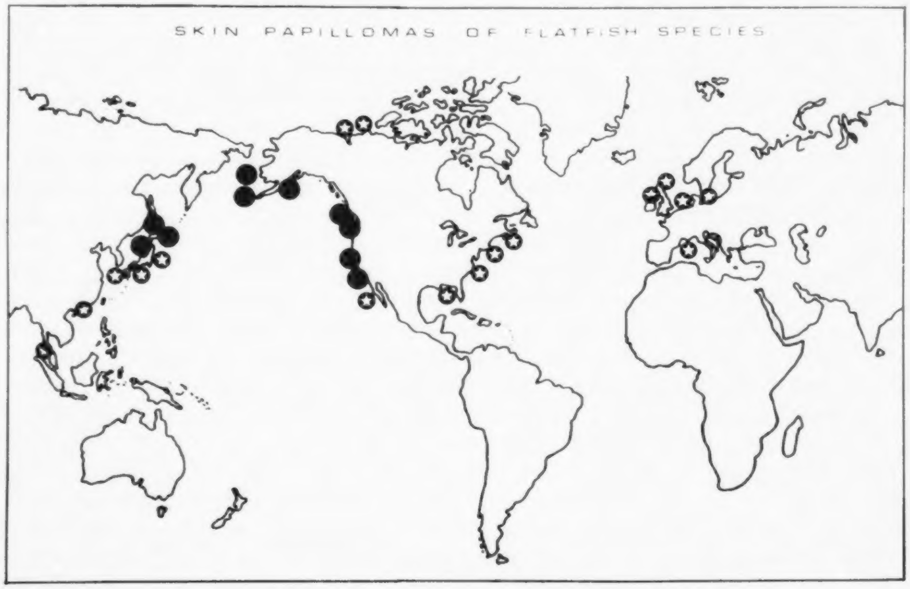

Figure 1. Distribution of flatfish species, *Pleuronectidae*, with skin papillomas (●). The examined species are listed by Stich *et al*. (1977b). The marked areas are free of skin papillomas (✪).

In spite of considerable effort, the cause(s) of the global distribution pattern has still eluded us. Previously, we have speculated about the involvement of a virus and the possibility that the papilloma cells result from an abortive lymphocystic infection in flatfish of the northern Pacific Ocean (Stich *et al.*, 1977a). However, there are other equally plausible explanations. For example, the global distribution pattern of skin papillomas could reflect the distribution of flatfish populations that are sensitive or resistant to the induction of skin neoplasms. Such an assumption would in turn raise the question as to the reasons why resistant fish species can evolve in one location and not at another.

Whatever explanation may finally prove to be correct, there is one important lesson to be learned from our global study on flatfish papillomas. An attempt to find a correlation between fish neoplasms and environmental agents should be preceded by a careful investigation of whether or not the selected fish species at the location under study is capable of developing a particular neoplasm. Such a preliminary exploratory study may avoid a lot of frustration. If feasible, we should even try to obtain *quantitative* information about the variations in sensitivity of fish populations from different geographic locations. Otherwise, there will always be the nagging suspicion that observed differences in tumor prevalences may reflect differences in sensitivity towards carcinogenic and mutagenic agents rather than differences in environmental contaminants.

Consistency of Tumor Prevalences

The prevalence of skin papillomas among flatfish populations varies markedly according to geographic location, season, and age group. At nursery grounds, a sampling period of about one year is required to gain accurate information on the peak of tumor prevalence. Considering the large geographic and age-dependent variations, it was somewhat unexpected that the prevalence figures of skin papillomas in flatfish populations did not change from year to year (Table 1). Even during longer periods, the peak prevalence was surprisingly constant, as exemplified by rock sole populations sampled off the Queen Charlotte Islands (Canada).

If tumor frequencies are to be used successfully in a monitoring program, this observed consistency in papilloma frequencies cannot be simply discarded, but should be explained. We could assume that a contaminated estuary does not rapidly change, and thus juvenile fish are exposed to similar carcinogenic levels for long periods. One of the more intriguing explanations would be to assume a genetic heterogeneity within the fish population, consisting of sensitive and resistant individuals and further, to postulate that their ratios differ among populations in different locations. The latter idea is by no means farfetched. For example, there appears to be a particular distribution of aryl hydrocarbon hydroxylase inducibility among the normal human population that apparently increases the risk of lung and laryngeal carcinomas (Kellermann *et al.*, 1973; Kellermann, 1977; Brandenburg and Kellerman, 1978). Again, the different sensitivities of various mouse strains to cancer induction are to a great extent based on the genetic control of metabolism, activation, and detoxification of carcinogens (Nebert and Felton, 1976; Nebert et al., 1976; Kouri and Nebert, 1977). But, even among fish (platyfish-swordtail system), the different responses to carcinogenic agents, including X-rays and N-methyl-N-nitrosourea (MNU), have been traced to various combinations of tumor and regulatory genes (Anders et al., 1971; Anders and Anders, 1978; Schwab et al., 1978a,b). If differing sensitivities to environmental carcinogens are really genetically based, then a balanced polymorphism may exist and subgroups with different ratios of sensitive and resistant fish could arise by selection. This selection could be by the action of carcinogens, and one could imagine a situation where increased death rate of sensitive individuals could produce a population with a lower tumor prevalence. Such an assumption could also explain the observation that the prevalence figures are relatively constant over a time period and that "constant" prevalence figures vary between populations of the species inhabiting different geographic locations.

TABLE 1. CONSTANCY OF SKIN PAPILLOMA PREVALENCES AMONG FLATFISH POPULATIONS SAMPLED AT DIFFERENT TIME PERIODS

Location	Species	Year of Sampling	(%) Prevalence Skin Papilloma
Queen Charlotte Islands, B.C.	*Psettichtys Melanosticus* (sand sole)	1953	33%
		1975	31%
Bay of San Francisco	*Parophrys vetulus* (English sole)	1968	29%
		1975	28%
Vancouver, B.C.	*Parophrys vetulus* (English sole)	1973	56%
		1974	58%
		1976	60%
Everett, Washington	*Parophrys vetulus* (English sole)	1975	44%
		1976	41%

TUMOR PREVALENCES AND B(a)P IN BOTTOM SEDIMENTS

Since flatfish stay for prolonged periods in their life span within layers of mud, it was expected that carcinogens in the bottom sediments could directly affect the surface fish, leading to the observed skin papillomas. It was further argued that, in such a case, a correlation between prevalence of skin papillomas and the level of carcinogen in the sediments of nursery grounds should become easily evident. Actually no simple link between neoplasms of flatfish and levels of benzo(a)pyrene (BaP) in bottom sediments was observed (Table 2). For example, relatively high levels of BaP occur in the Jade (Northern Germany) or near Hong Kong, but not a single skin papilloma was seen among six local flatfish species. But even within the northern Pacific, which we previously called an area of potential risk regarding skin papilloma risk (Stich et al., 1977a), no simple quantitative relationship between BaP and skin papillomas could be established (Table 2). The high prevalence of skin papillomas found among fish populations in the vicinity of urban and industrial centers (Stich and Acton, 1976; Stich et al., 1976a,b; 1977a,b) cannot be due only to PAH. Moreover, it would be misleading to assume that all PAHs originate from man-made activity. The fairly high levels of BaP at Furen Lake in Hokkaido and at the MacKenzie delta and surrounding coast are likely to be due to naturally occurring phenomena, such as forest fires, which in the northern regions may contribute a greater amount of PAH to the environment than generally assumed (Beumer and Youngblood, 1975; Lunde and Bjorseth, 1977; McMahon and Tsoukalas, 1978). PAHs can also be transported over wide areas (Lunde and Bjorseth, 1977) and may have, in cold arctic or subarctic waters, different rates of biodegradation (Atlas, 1977) that could lead to their accumulation over longer time periods.

Numerous reasons can be cited in an attempt to explain the lack of an obvious correlation between skin papilloma prevalences and BaP levels in bottom sediments: (a) PAHs are potent skin carcinogens for mammals, but may not have a similar effect on fish skin; (b) the concentration of PAHs is too low; (c) PAHs are bound to particulate matter within the bottom sediment and thus do not enter the fish skin; (d) BaP and other PAHs do not represent a major part of carcinogenic compounds in marine bottom sediments.

Before the question of involvement in induction of tumors of marine organisms is discarded or downgraded, the measurements of BaP and other carcinogenic compounds in the marine environment should be critically reviewed. Is it valid to assume that bottom sediments are the source of PAHs that enter bottom-dwelling or free-swimming organisms? If PAHs pass into invertebrate and vertebrate via the food chain, then the BaP content of the biotope of the food chain organisms is more crucial than that of the indicator organisms. Thus PAH measurements of the bottom sediment could easily be misleading. At present it is difficult to assess whether sediments properly reflect the PAH levels at the water surface or PAH in suspension. The use of several accummulator organisms that inhabit a variety of habitats will lead to a more complete evaluation of the PAH actually available to living organisms (Mix et al., 1977).

BaP in Bottom Sediments and AHH Activity in Flatfish

Aryl hydrocarbon hydroxylase (AHH) is involved in the activation and detoxification of a wide array of carcinogens. In rodents and man, the inducibility of this enzyme appears to be genetically controlled, thus dividing many populations into responsive and nonresponsive subgroups with a high and low sensitivity to tumor induction, respectively (Kellermann et al., 1973; Nebert and Felton, 1976; Nebert et al., 1976; Kellermann, 1977; Kouri and Nebert, 1977; Poland and Kende, 1977; Boulos, 1978). AHH activity is also found in fish (Lee et al., 1972; Payne and Penrose, 1975; Payne, 1976; Bend et al., 1977). If PAHs comprise a major component of carcinogens in the marine sediments, we should expect to find a correlation between BaP levels and AHH activity of bottom-dwelling fish. Such a correlation was actually observed by plotting BaP levels against AHH levels in livers of lemon soles sampled at different locations off the coast of

TABLE 2. THE CONTENT OF BaP IN BOTTOM SEDIMENTS AND THE PREVALENCE OF SKIN PAPILLOMAS AMONG FLATFISH AT VARIOUS LOCATIONS

Location	B(a)P µg/kg dry weight	Prevalence(%) Skin papillomas	Species and References
Wilhelmshaven			
sand	35-1660	0	
silt	4- 51		
Hong Kong			
sand	2- 38	0	11 species**
Kotzebue	0.4-0.5	0-23	*Lepidopsetta bilineata****
Shesbalik	0.1-2.2	0	5 species***
Baldwin Peninsula	0.2-1.9		
Prince of Wales	<0.1-1.1		
Nome	<0.1-0.8		
Seward	0.4		
Kittigazuit	1.8/24.3	0 0	*Liopsetta gracialis* *Platichthys stellatus*
Kings Point	0.6-17.3		
Kay Point	117		
Shingle Point	0.3/6.3		
MacKenzie Delta	2-33		
Furen Lake	55*	33	*Limanda schrenki*
Shore of Okhotsk Sea	0.2*	0.3	*Limanda schrenki*
Everett	0.3	42-44	*Parophrys vetulus*
Port Susan	0.7	20-26	*Parophrys vetulus*
Utsaladdy	0.6	1- 3	*Parophrys vetulus*
Vancouver		56-60 0 0	*Parophrys vetulus* *Platichthys stelatus* *Microstomus pacificus*
Port Renfrew	11.1	0 0	*Parophrys vetulus* *Platichthys stelatus*

*B(a)P µg/kg wet weight
**Stich *et al*., 1977a
***Wellings *et al*., 1977

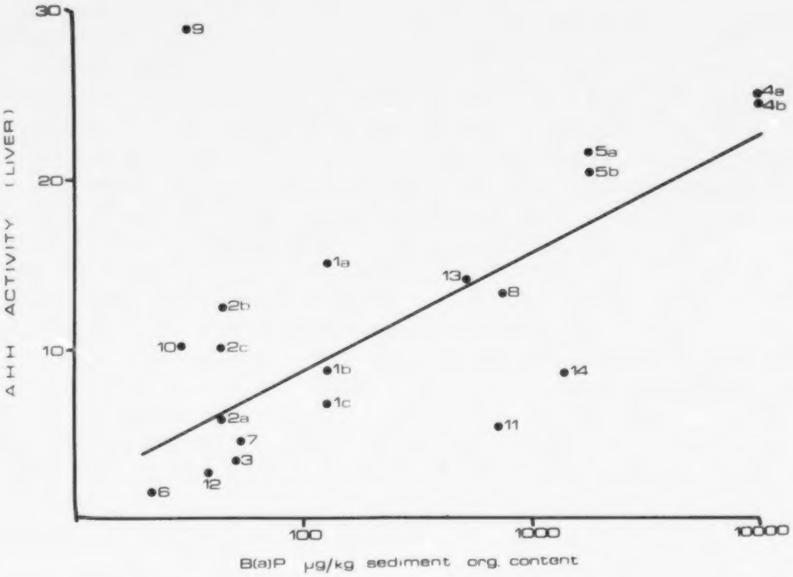

Figure 2. Correlation between BaP levels in bottom sediments and AHH activity (p mole/min/mg/liver) in the liver of lemon sole sampled at the following locations in British Columbia and the State of Washington: 1. Vancouver, 2. Gibsons, 3. Crescent Beach, 4. Coal Harbor, 5. Port Moody, 6. Denman Island, 7. Tofino, 8. Port Renfrew, 9. Everett, 10. Port Susan, 11. Henderson Inlet, 12. Utsaladdy, 13. Bellingham, and 14. Olympia. Per point between 2 and 12 bottom samples were analyzed according to the method of Dunn (1976), Dunn and Stich (1975), and Dunn and Young (1976).. AHH was assayed following the procedure of DePierre _et_ _al_. (1975).

British Columbia and Washington (Figure 2). As expected, some fish populations did not fit into the overall picture. An exceptionally high AHH activity was found in the liver of flatfish collected near a paper mill in an area with a relatively low BaP concentration. The observed correlation seems to be in good agreement with reports on elevated AHH activity in livers and gills of the cunner, Tautogolabrus adspersus, in oil-contaminated bays of Newfoundland (Payne and Penrose, 1975; Payne, 1976). However, a correlation can only be seen when relatively large numbers of flatfish are examined and the fish populations are sampled at the same period of the season. Even when age, size, and weight are adjusted, and the fish are collected in one small territory from a uniform type of bottom sediments, the AHH activity can vary considerably (Figures 3,4,5). This variation in AHH activity becomes particularly evident if flatfish are collected from offshore areas contaminated by complex mixtures of urban and industrial effluents (Figure 3). On the other hand, the AHH levels can be quite similar. This pattern is frequently encountered in areas without any obvious pollution by industrial, urban, or agricultural discharges. Again, we would like to point out that a simple correlation may either not exist or be found only in exceptional cases with large differences between pristine and contaminated areas. As seen in Figure 4, even within the same habitat, different species of comparable age can greatly differ in their liver AHH activities. Of particular interest is that the variations in AHH activities of 1-, 2-, and 3-year-old lemon soles inhabiting the same contaminated environment did not significantly differ (Figure 3.)

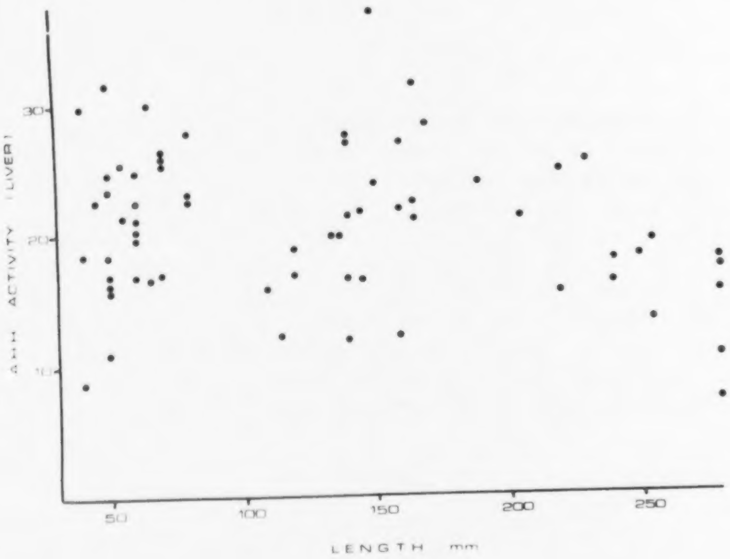

Figure 3. AHH activities in the liver of lemon sole of various lengths sampled at Port Moody. A point represents the AHH activity in the liver of our fish.

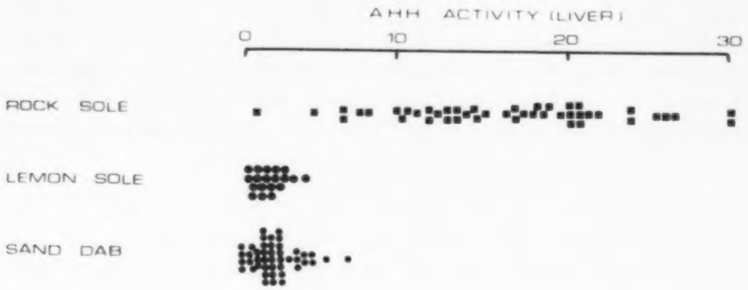

Figure 4. AHH activity in the liver of two sole species and sand sole collected off Denman Island.

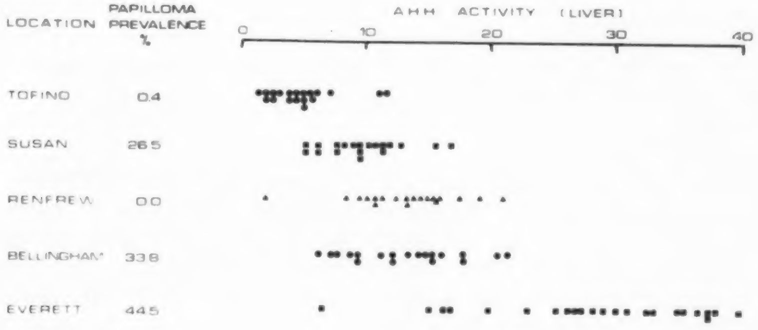

Figure 5. AHH activity of the liver of lemon sole collected at several locations which differed in their skin papilloma prevalences.

AHH Activity in Flatfish and Tumor Prevalence

The possibility of a positive or negative correlation between AHH levels in the livers of flatfish and tumor prevalence was examined. First, we measured the enzyme activity of lemon sole populations that differed greatly in their prevalences of skin papillomas. As seen in Figure 5, populations with high prevalences of skin tumors can have low or high AHH levels. Then, we compared the AHH levels of tumor-bearing lemon soles with those in tumor-free fish of the same age group and from the same location. No significant difference between these two fish groups was observed.

Outlook

Based on our experience with indicator organisms, we would like to comment on possible future developments of this attractive, but as yet unproven approach to assessing environmental hazards to man or to animal populations. Foremost on a list of priorities should be the introduction of genetically well-defined organisms as subjects in examining the carcinogenic or mutagenic load of the marine environment. Although this suggestion may smack of heresy, the fact that our current progress in the use of lower vertebrates or invertebrates in detecting carcinogens lags far behind the considerable advances made with inbred strains of rodents cannot be ignored.

The use of wild, randomly mating fish populations for examining the carcinogenicity or mutagenicity of compounds would be comparable to performing tests on field mice collected from different geographic areas--a situation which would be discarded as totally useless. The selection of highly sensitive strains of bacteria, yeast, and molds for the numerous short-term tests to detect the mutagenic properties of compounds or complex mixtures is another example which must be emulated if we are to become successful in introducing marine or fresh water organisms as test subjects. The selection of genetically well characterized subjects with known sensitivity to one or another group of obnoxious compounds appears to be a prerequisite to establishing the use of a marine organism at a sound scientific level.

The opinion has been repeatedly expressed that the ultimate judgment about the carcinogenic hazard of an environment can only be obtained by establishing tumor prevalences of the population under discussion and its subgroups. This would mean that only a human population could provide any indication as to whether or not a particular environment is carcinogenic for man. Although one cannot deny the validity of such an approach, the objective of monitoring programs, early-warning systems, and analysis of contaminations is to prevent such an occurrence. The question is not whether predictive tests should be introduced into the repertoire of preventive medicine, but rather which of the numerous biological and chemical procedures can best reflect the carcinogenic or mutagenic hazard in a marine environment. At present, chemical analysis cannot reveal the carcinogenic/mutagenic property of a compound. Microbial short-term bioassays can detect the mutagenic capacity of a compound, but whether a positive result indicates that a compound is carcinogenic to, for example, man, rodents, fish, or oysters, is a debatable issue. Thus, tumor frequencies of indigenous indicator organisms could represent a highly promising approach when the sensitivities and responses of these organisms to particular carcinogens or complex carcinogenic mixtures become known. Such information could be readily obtained by properly designed experiments under controlled laboratory conditions.

We would be remiss if we listed only the difficulties encountered in the use of indicator organisms and omitted the encouraging observations. For example, a study of tumor prevalences along the shores of Hokkaido has revealed an exceptionally high value (33%) among flatfish in one shallow, poorly flushed bay, which is in a region devoid of industry, urban activity, or agricultural usage. Subsequent analysis of BaP in the bay has shown concentrations in the order of 55 µg/kg, whereas areas open to the sea had low BaP concentrations, and their flatfish population also had a low frequency of neoplasms. This pattern seems to exemplify how even preliminary data on tumor frequencies can help to detect hotspots of contamination. But much more impressive in its predictive value was the discovery that tumor-like growth anomalies appeared among cultured algae, Porphyra tenera, Nori, at the mouth of particular rivers in Kyushu, Japan (Ishio et al., 1970, 1972a,b). The distribution pattern of algae "tumors" combined with tumor induction experiments and chemical analyses led to the source of contamination: PAH-releasing industrial complexes. But probably the most convincing evidence in favor of the indicator organism was the gradual disappearance of the tumor-like growth anomalies following the introduction of preventive measures by the factories responsible. This example seems to rank in importance equal to the discovery of the carcinogenic potential of aflatoxins by investigating outbreaks of hepatomas among trout kept at particular hatcheries and fed a particular contaminated diet. Other fascinating, but less well-documented results have been reported from China where chickens developed esophageal tumors in regions in which a high frequency of esophageal carcinomas also occurred in man. Pigs also have been found with nasopharyngeal tumors in districts with comparable carcinomas among the human population (Lawrence, 1977).

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POLYNUCLEAR AROMATIC HYDROCARBONS IN ESTONIAN WATER, SEDIMENTS, AND AQUATIC ORGANISMS

by

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ABSTRACT

Benzo(a)pyrene (BaP) was selected as a model polynuclear aromatic hydrocarbon in a survey of Estonian marine and freshwater water column, sediments, algae, aquatic plants, and fish. Water samples had low BaP content; sediments were much higher. Water samples taken in the summer season had the lowest BaP content. Fish livers had higher BaP residues than gills and gonads contents. Weight class of fish was not correlated with BaP residues in the case of herring. However, large predatory fish had lower residues than medium-sized predatory fish. Species of fish with high fat content had higher residues of BaP than low-fat fish.

INTRODUCTION

Estonia has over 1500 lakes, 7000 rivers, and 3780 kilometers of coastline that provide important water resources for freshwater and Baltic Sea fisheries, agar harvests for use in ice cream and other foods, recreation, and a source of drinking water from lakes and rivers.

Many research papers have shown that polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the aquatic environment and that some are carcinogenic. Benzo(a)pyrene (BaP) has been used as an indicator of the diverse group of PAHs because of its known carcinogenic activity, stability, frequency of occurrence, and ease of chemical analysis. There are no previously reported studies on the occurrance of BaP in the aquatic environment of Estonia.

MATERIAL and METHODS

Our preliminary survey was undertaken to determine the distribution of BaP in water, sediments, and tissue residues of various aquatic organisms. Sampling sites selected were of economic significance and represented different types of water bodies (Maemets and Raitviir, 1977). Most of the sampling was undertaken at least once per season, but some lakes were sampled only during the summer. Over 2000 samples were analyzed from 64 lakes, 21 rivers, and 8 bays of the Baltic Sea. Fish samples were collected from 22 species; more than 400 samples were analyzed.

BaP was determined by the following method: 3 ℓ water was repeatedly extracted with ethyl ether; 100 mℓ aliquots of the solvent were successively used to extract each water sample. The extractions were pooled, steam distilled to dryness, and extracted with n-octane. BaP was determined by quasi-linear luminescent spectra using a modification of the method of Khesina (1968). Samples were read at - 196° C in solid parafins.

Fish residues were analyzed for BaP as follows: organs or fillets were homogenized, 100 g homogenized tissues were immersed in 100 mℓ ethyl alcohol, and 15 to 25 g KOH added. Then the mixture was boiled for 2 hr. The resultant mass was immersed in water and extracted five times with ethyl ether. The extract was washed initially with acidized water (5% H_2SO_4) and finally with distilled water. NA_2SO_4 was added to the extract, and the ether was removed by steam distillation. The residue was dissolved in n-octane and read as in the water analyses procedure.

Water plants were extracted in benzene with a Soxleth apparatus. The benzene was removed by steam distillation and the residue dissolved in n-octane. The BaP in n-octane was separated by thin layer chromatography, using several solvent systems. The fluorescent front was scraped off the plate, eluted with benzene, and then measured by luminescence as in water samples. The fish used in this study are described in Table 1.

RESULTS

Table 2 summarizes the BaP content of water, sediments, and aquatic organisms. All water samples had BaP concentrations below the USSR sanitary limit (0.005 μg/ℓ water). Seawater samples contained slightly more BaP than freshwater samples; the lowest concentrations were found in lakes and rivers of non-industrialized areas (Veldre *et al*., 1977; 1979). During summer, BaP content was lower than in winter when lakes in Estonia are covered by ice. Although water concentrations were low, BaP concentrations were high in saltwater and freshwater sediment (Bogovski *et al*., 1977). Freshwater aquatic plants had exceptionally high concentrations of BaP in tissue residues.

TABLE 1. CHARACTERISTICS OF FISH STUDIED

Fish	Species	Water	Fat Content
Carp	*Cyprinius carpio*	Freshwater	high
Seatrout	*Salmo trutta trutta*	Saltwater Freshwater	high
Tench	*Tinca tinca*	Freshwater	high
Salmon	*Salmo salar*	Salt & Freshwater	high
Pike	*Esox lucius*	Freshwater	low
Eel	*Anguilla anguilla*	Fresh & Saltwater	high
Perch	*Perca fluviatilis*	Fresh & Saltwater	low
Roach	*Rutilus rutilus*	Freshwater	low
Bream	*Abramis brama*	Freshwater	high
Pike-perch	*Lucioperca lucioperca*	Fresh & Saltwater	low
Baltic sprat	*Sprattus sprattus balticus*	Saltwater	high
Baltic herring	*Clupea harengus membras*	Saltwater	low
Silver bream	*Vimba vimba*	Salt & Freshwater	low
White bream	*Blicca bjoerkna*	Freshwater	low
Brown trout	*Salmo trutta*	Freshwater	high
Minnow	*Phoxinus phoxinus*	Freshwater	low

TABLE 2. LEVEL OF BaP IN WATER, SEDIMENTS, AND AQUATIC ORGANISMS

Source	Sample	Number of Samples	Concentration (μg/kg,and μg/ℓ, respectively) min	max	arithmetic mean
Saltwater	water	29	<0.0000	0.0123	0.0007
	sediments	16	0.24	11.50	2.57
	algae*	2	2.40	3.00	2.70
	zooplankton**	13	0.26	5.10	1.11
	baltic sprat	23	0.22	4.48	0.99
	baltic herring	12	0.08	3.10	1.22
	pike-perch	9	0.06	8.50	2.25
Freshwater	water	32	<0.0000	0.0047	0.0006
	sediments	2	2.10	4.30	3.20
	waterplants				
	reed(*Phragmites*)	10	1.32	129.0	36.90
	duckweed (*Lemna*)	3	19.10	112.4	52.40
	roach	6	0.03	3.04	0.73
	perch	8	0.02	1.90	0.83

* Collected by hand with aqualung

** Collected by plankton tongs using a silk net No. 39

Table 3 summarizes the BaP content in fresh and saltwater fish fillets. Most fish contained 1 to 2 μg/kg BaP (10 to 20 μg/kg on a dry weight basis). Some fish (salmon, eel) contained 5 to 10 μg/kg BaP. Among the freshwater fish tested, the pike, minnow, and pike-perch had low tissue residues as compared to eel, salmon, and some saltwater species. Table 4 lists the BaP content in muscular tissue of predatory and non-predatory fish. The results showed no marked difference in the BaP content of the fish. Table 5 demonstrates that fish livers have higher BaP residues than gills or roe and, in some cases, fish fillet.

Figure 1 summarizes the BaP content in low- and high-fat fish. Species of fish with high-fat content have higher residues of BaP than low-fat fish. The relationship of BaP tissue residues to the weight of fish is illustrated in Figure 2. Weight class of fish is not correlated with BaP residues in the case of Baltic herring. Large (700 to 1100 g) predatory fish (pike-perch and tench) have lower residues than medium-sized (400 to 600 g) fish.

TABLE 3. CONTENT OF BaP IN THE MUSCULAR TISSUE OF FISH

Source	Species	Number of Samples	Concentration µg/kg		
			min	max	arithmetic mean
Rivers of North Estonia	trout	4	< 0.01	1.72	0.685
	minnow	2	0.17	0.19	0.180
	salmon	2	0.11	11.82	5.960
Various lakes	tench	8	0.09	2.94	0.962
	pike	11	0.05	0.41	0.187
	eel	7	0.00	24.00	4.370
	perch	8	0.02	1.90	0.835
	roach	6	0.03	3.04	0.733
	bream	32	<0.01	10.00	1.694
	pike-perch	4	0.23	0.76	0.417
Bays and gulfs	baltic sprat	23	0.22	4.48	0.99
	baltic herring	12	0.08	3.10	1.22
	pike-perch	9	0.06	8.50	2.25
	perch	2	0.10	0.52	0.31
	silver-bream	2	0.08	0.13	0.11
	white bream	2	0.20	0.48	0.34

TABLE 4. THE LEVEL OF BaP IN PREDATORY AND NON-PREDATORY FISH

Fish	Number of Samples	Concentration µg/kg min	max	arithmetic mean
Predatory				
pike	11	0.05	0.41	0.187
pike-perch	4	0.23	0.76	0.417
trout	4	< 0.00	1.72	0.685
eel	7	< 0.00	24.00	4.370
Non predatory				
bream	32	< 0.00	10.00	1.694
tench	8	0.09	2.94	0.962
roach	6	0.03	3.04	0.733

TABLE 5. THE COMPARATIVE CONTENT OF BaP IN VARIOUS ORGANS OF FISH

Fish	Arithmetic mean of BaP concentration (µg/kg) Gills	Liver	Roe	Fillet
tench	0.16	0.47	0.97	0.962
pike	0.15	0.75	-	0.187
bream	0.82	0.59	0.08	1.694
herring	0.44	1.69	1.13	1.220

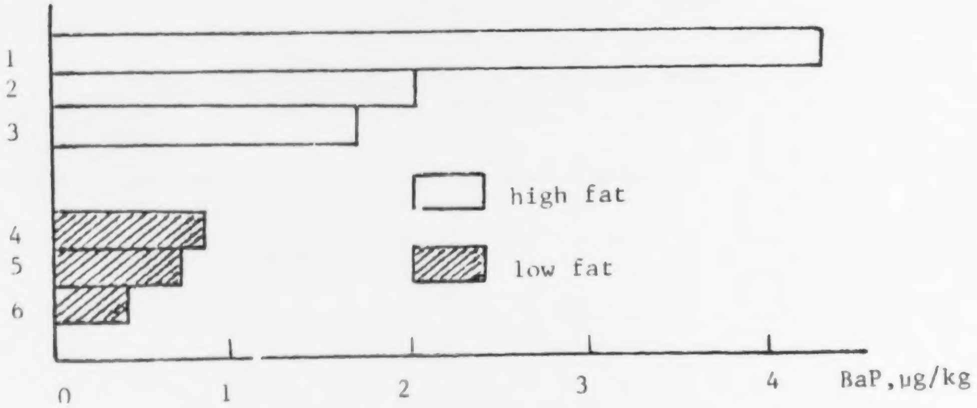

Figure 1. Relationship of BaP tissue residues in high and low fat fish.

1 - eel	4 - perch
2 - tench	5 - roach
3 - bream	6 - pike

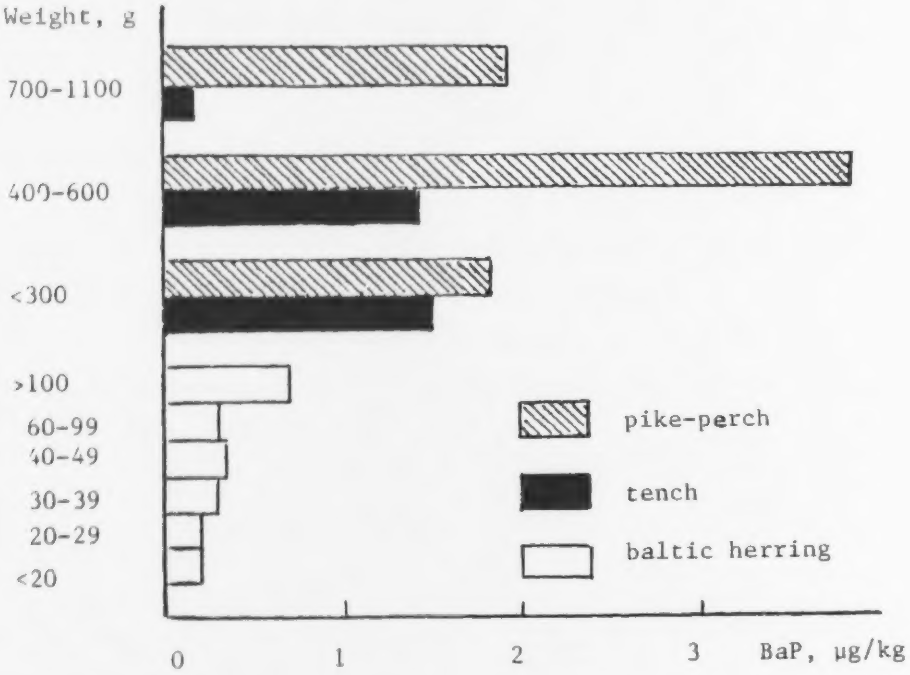

Figure 2. Relationship of BaP tissue residues to weight of fish.

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BIOACCUMULATION AND TOXICITY IN ENGLISH SOLE PAROPHRYS VETULUS FOLLOWING WATERBORNE EXPOSURE TO BENZO(A)PYRENE

by

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ABSTRACT

Juvenile English sole, Parophrys vetulus, measuring 10 to 30 cm in total length were held in an all glass/teflon aquarium system at 11° C and exposed continuously for a 30-day period to artificial seawater containing low levels of benzo(a)pyrene (BaP). The BaP was present both in solution [(1.0 parts per billion (ppb)] and as crystals coated onto the sand substrate. At the termination of the test, the fish were examined to determine the level of BaP in the tissues, the level of hepatic aryl hydrocarbon hydroxylase (AHH) activity, and the extent of tissue damage.

Gas chromatographic analysis revealed detectable levels of BaP in tissue extracts as well as markedly significant quantities adsorbed onto integumental surfaces. Awareness of this adsorption phenomenon is critical to an understanding and an accurate determination of whole body uptake. Enzyme analysis by fluorescence spectroscopy using pooled data indicated that mixed function oxygenase activity in the experimental fish was increased by over 100%. However, closer inspection of individual enzyme levels showed wide variability and a bimodal pattern of AHH activity. Histopathological examination revealed the presence of free blood in either the pericardial or abdominal cavities in 55% of the test fish. This change was not noted in control animals. In addition, the exposed fish had increased numbers of immature white blood cells in peripheral circulation.

INTRODUCTION

Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon which has frequently been selected as a model compounmd for studies on the fate and effects of this ubiquitous group of chemicals. BaP occurs in such media as cigarette smoke, automobile exhaust, coal tar, and soot (IARC, 1973), and resultant environmental contamination by this compound has been noted in air, vegetation, fresh and salt water, food, and soil (IARC, 1973). It is not surprising therefore that significant bioaccumulation of BaP has been noted in several aquatic organisms (Neff and Anderson, 1975; Dunn and Stich, 1976; Varanasi and Malins, 1977).

BaP is known to be carcinogenic in a variety of homeothermic animals (IARC, 1973), but little is known of its toxic effects or mutagenic potential in marine poikilotherms (Hodgins *et al*., 1977). The experiment herein reported represents an interdisciplinary approach to assessing such effects and uses the English sole, *Parophrys vetulus*, as a model species.

English sole are members of the family Pleuronectidae. They begin life as pelagic eggs, live for a time as littoral larvae, and, after metamorphosis, become benthic organisms living in intimate contact with sediments; thus, in the course of their development these fish are exposed to all levels of the water column. They have world-wide distribution, are not migratory, and their diet includes, among other things, bivalve molluscs which are capable of concentrating and storing environmental contaminants such as BaP (Lee *et al*., 1972a; Fossata and Canzonier, 1976).

In our study juvenile English sole were exposed for 30 days to chronic low levels of waterborne BaP. At test termination, the fish were analyzed to determine whether bioaccumulation of BaP had occurred, whether hepatic BaP hydroxylase activity was altered, and whether tissue lesions were present.

MATERIALS AND METHODS

Collection and Housing of Fish

Fifty juvenile English sole 10 to 30 cm in total length were collected by beach seine at Eagle Cove, San Juan Island, WA. The fish were transported to the University of Washington, School of Fisheries, where they were placed in five 230-ℓ aquaria. These holding tanks contained artificial seawater and were part of a closed aquarium system constructed entirely of glass and teflon as developed for toxiological studies. The fish were held at 11° C and allowed to acclimate for one month prior to testing. The actual exposure period lasted for 30 days.

Addition to and Quantitation of BaP in Seawater

Fine sand was washed with soap and water, then rinsed with water and acetone, and baked dry. Purified BaP was dissolved in methylene chloride, coated onto the sand (500 mg BaP/kg sand), and the methylene chloride was subsequently evaporated under a stream of nitrogen. To each of four aquaria was added 750 g of the BaP-coated sand. In addition, 2 kg of cleaned coarse sand, similarly coated, was packed into glass tubing and placed in the water delivery system of the four experimental aquaria. Only clean sand was placed in the control tank.

The concentration of dissolved BaP was monitored throughout the test period by the following procedure. A known volume of water (50 mℓ) was filtered through methylene-chloride-washed filter paper, then placed in a 250 mℓ separatory funnel, and extracted three times with 25 mℓ of reagent grade methylene chloride. The organic solvent portions were subsequently combined, made up to a known volume (100 mℓ), and adequately mixed. An aliquot of the solution was analyzed on a Perkin-Elmer MPF fluorescence spectrophotometer (excitation wavelength 365 nm, slit 10 nm, spectrum scanned 340 to 500 nm). Maximum emission was obtained at 405 nm, and the height of the peak was used to quantitate the amount present. Large quantities of crystalline BaP were in circulation in the system; however, due to its non-polar nature only about 1.0 ppb BaP was actually in solution at any given time. Filtration of the water prior to analysis allowed for removal of the crystals and for accurate determination of water-borne concentrations.

Quantitation of BaP Tissue Levels by Gas/Liquid Chromatography

Fish were sacrificed by a blow to the head and then frozen for analysis. Before analysis, each fish was washed with methylene chloride to remove any crystalline BaP adsorbed onto integumental surfaces. The entire animal was then homogenized. A portion of the tissue was accurately weighed and placed in a flask containing 100 mℓ 0.5 M KOH in methyl alcohol, 100 mℓ contaminant-free distilled water, and boiling stones. The mixture was refluxed for 18 to 24 hr. After saponification, the mixture was transferred to a 1-ℓ separatory funnel containing 100 mℓ nanograde hexane, 35 mℓ nanograde toluene, and 100 mℓ solution of saturated Na_2SO_4 in distilled water. The funnel was shaken for 2 min, the layers allowed to separate, and the hexane-toluene layer decanted into a clean 100-mℓ flask. The remaining aqueous-methanol phase was subsequently partitioned with two additional aliquots of 100 mℓ hexane plus 35 mℓ toluene, and all three hexane-toluene aliquots were combined. The mixture was then reduced to a small volume (3 to 5 mℓ) on a rotary evaporator and quantitatively transferred to a 25-mℓ mixing cylinder with hexane toluene rinses.

Between 0.2 and 0.5 g of non-saponifiable fraction was dissolved in no more than 5 mℓ hexane and transferred to a chromatograph column (22 mm I.D.) containing 16.5 cm Florisil and an upper 1.2-cm layer of anhydrous Na_2SO_4; 300 mℓ hexane was passed through the column at a flow rate of 10 mℓ/min followed by 300 mℓ of a 30% (v/v) mixture of methylene chloride in hexane at the same flow rate. The volume of this fraction was reduced on a rotary evaporator, transferred quantitatively to a glass vial, and reduced to dryness with a stream of N_2 gas.

Gas liquid chromatographic analysis was performed on the Hewlett-Packard 402 high performance gas chromatograph under the following conditions: Column: 6 ft x 1/4 inches O.D. all glass column
Packing: Supelco SP 2350 on acid-washed 100/120 chromosorb W
Carrier Gas: N_2, 50 mℓ/min, Rotameter setting 4.0
Hydrogen: 35 mℓ/min, Rotameter setting 3.5
Air: 20 mℓ/min, Rotameter setting 3.5
Temperature Column: 255° C, detection 290° C, injector 280° C
Range 1, attenuation 16 or 30 X

Histopathological and Hematological Analysis

Approximately 1 mℓ of blood was withdrawn into a heparinized syringe by caudal vein puncture of unanesthetized animals. The fish were then sacrificed by immersion in Bouins solution, and the abdominal cavity was injected with more fixative. After 24 hr, tissues were washed and transferred to 70% ethanol.

Serial whole body cross sections were dehydrated in a graded series of ethanols, cleared in xylene, and embedded in paraffin; 6-μ sections were prepared using a rotary microtome and were stained with hematoxylin and eosin. Smears were prepared from the heparinized blood and stained with Leishman and Giemsa stains. Capillary tubes were filled with a portion of the remaining blood for hematocrit determination.

Hepatic Aryl Hydrocarbon Hydroxylase Analysis

The fish were sacrificed by a blow to the head and the liver was immediately removed *in toto*, weighed, diluted 1:23 (wt:vol) in cold 0.15% KCl, and homogenized. The homogenate was placed in a refrigerated (4° C) centrifuge and spun for 20 min at 100,000 rpm (9000 x g), and the clear supernatant fluid was collected.

The supernatant fluid was next incubated for 20 min at 19.5° C in a mixture of Tris buffer (0.1 M pH 7.2), MgCl (33 mM), distilled water, BaP (0.4 mg/mℓ in methanol), and NADPH (8 mg/mℓ). The reaction was stopped by the addition of 1 mℓ cold acetone, and the mixture immediately extracted by constant shaking (25° C) for 10 min in 3.25 mℓ reagent grade hexane. A portion of the hexane layer (1 mℓ) was decanted, placed in 3 mℓ cold NaOH, and extracted for 5 min at 25° C with constant shaking (Nebert and Gelboin, 1968).

The NaOH layer was then analyzed on a Perkin-Elmer MPF spectrofluorimeter (396 nm excitation, 522 nm emittance) and the results expressed in fluorescence units (Fu)/mg protein. The protein determinations were performed according to the method of Lowry (Lowry *et al*., 1957).

RESULTS

At the termination of the 30-day exposure, all of the fish appeared to be in generally good health and were feeding regularly. Of 40 experimental fish, 32 survived. No control animals died during the test.

Levels of BaP in Seawater

BaP is a non-polar compound which is sparingly soluble in seawater (20 ng/mℓ saturation) and which, due to its hydrophobic nature, is difficult to maintain in solution. Sufficient quantities of BaP were added to our system to achieve supersaturation; however, upon letting the water stand for a few hours, the amount of BaP in solution declined to less than 1 ppb and persisted at that level throughout the experiment. Large quantities of crystalline BaP were present in the aquaria at all times as evidenced by filtration prior to water analysis.

Uptake of BaP into Fish Tissue

No BaP was found in the three control fish analyzed (Table 1); however, measurable levels were found in 10 of 11 experimental animals. Fish 1 through 5 were analyzed without benefit of prior washing and yielded whole body accumulations of from 137 to 282 ppb in test animals (Table 1).

BaP has a strong tendency to adsorb onto surfaces, such as glass aquarium walls and sand substrate. Because of this phenomenon, the integuemental surfaces of fish 6 through 14 were washed repeatedly with methylene chloride, without scraping, to remove loosely bound BaP. Table 2 shows that significant amounts of BaP were removed in the course of these washings. This material represents superficial adsorption rather than true uptake.

Table 1 shows the uptake of BaP by fish 6 through 14 following removal of the compound from integumental surfaces. High individual variability was noted with values ranging from undetectable levels to a high of 499 ppb. Such discrepancies in the amount of parent compound present in tissues may have related to differential capacity to metabolize BaP (see AHH activity section).

HEPATIC AHH Activity

At test termination, 11 experimental and four control fish were sacrificed for analysis of hepatic AHH activity. On the basis of pooled data, the mean level for the experimental fish was 57.9 Fu/mg protein as compared with 22.3 Fu/mg protein for control animals (Table 3). Thus, an increase of over 100% in enzyme activity appeared to occur following continuous exposure to BaP.

TABLE 1. UPTAKE OF BaP BY ENGLISH SOLE FOLLOWING 30-DAY WATERBORNE EXPOSURE

Fish Number	Total Weight of Fish (g)	Wet Weight Analyzed (g)	Total Amount of BaP in Tissue Analyzed (ng)	Concentration (ppb)
Unwashed				
1 (exptl)	77.9	40.1	5,500	137
2 (control)	15.0	11.0	N.D.	N.D.
3 (exptl)	40.6	34.8	7,150	205
4 (control)	18.5	16.1	N.D.	N.D.
5 (exptl)	50.0	38.2	10,770	282
Washed				
6 (control)	20.1	15.3	N.D.	N.D.
7 (exptl)	10.2	7.2	270	37
8 (exptl)	5.6	5.3	690	130
9 (exptl)	31.3	21.4	810	38
10 (exptl)	43.3	33.1	16,530	499
11 (exptl)	6.9	5.4	30	N.D.
12 (exptl)	11.2	8.7	3,230	371
13 (exptl)	4.9	4.9	2,240	457
14 (exptl)	69.6	60.0	1,710	28

TABLE 2. CONCENTRATION OF BaP FOLLOWING WASHING WITH METHYLENE CHLORIDE OF INTEGUMENTAL SURFACE OF ENGLISH SOLE FOLLOWING 30-DAY WATERBORNE EXPOSURE

Fish Number	Total Weight of Fish (g)	Total BaP found in Extract (ng)
6 (control)	20.1	N.D.
7 (exptl)	10.2	285
8 (exptl)	5.6	414
9 (exptl)	31.3	230
10 (exptl)	43.3	395
11 (exptl)	6.9	993
12 (exptl)	11.2	4,450
13 (exptl)	4.9	15
14 (exptl)	69.6	1,695

TABLE 3. HEPATIC AHH ACTIVITY IN ENGLISH SOLE FOLLOWING 30-DAY EXPOSURE TO WATERBORNE BaP

	Control Fish	Activity FU/mg Prot.	Experimental Fish	Activity FU/mg Prot.
	1	18.2	1	17.3
	2	42.9	2	16.7
	3	7.9	3	2.3
	4	20.3	4	209.1
			5	5.9
			6	2.4
			7	42.3
			8	146.5
			9	10.3
			10	179.3
			11	5.0
MEAN		22.3		57.9

Closer inspection of the data revealed that wide individual variation occurred among the test animals, the level of enzymes varying from 2.3 to 209.1 Fu/mg protein. If the three fish with highest activities (Nos. 4, 8, and 10) were eliminated, the mean level of AHH activity among the experimental fish (12.8 Fu/mg protein) would be significantly decreased to a point even lower than that of the controls.

Since the compound was administered via a waterborne route rather than by feeding, all of the experimental animals should have been exposed to comparable toxicant levels. Thus, it appeared that the population of fish tested fell into two categories on the basis of BaP hydroxylase activity: those that were capable of responding to induction and those that were not.

Histopathology

In general, the animals were in excellent health. It was not possible to reliably distinguish treated from the untreated fish, because most of the lesions noted were common to both groups. None of the fish contained abdominal fat, although many had food in the lumen of the gastrointestinal tract.

Parasitism was noted in all the fish and consisted primarily of external infestations by the monogenetic trematode, Gyrodactylus sp., and of internal infestation by an unidentified microsporidan found both inter-

and intracellularly in the skeletal muscle of the body wall and intestine. Little inflammatory response was generated toward the organisms, which are common parasites of Puget Sound flatfish in their natural environment.

Limited numbers of fish in both groups evidenced subacute dermatitis apparently of bacterial origin. These lesions were characterized by epidermal erosions, peripheral epithelial hyperplasia, dermal inflammatory infiltrates, hemorrhage, edema, and limited involvement of underlying muscular tissue. Bacterial colonies were prominent and located primarily in dermal connective tissue.

Hepatic vacuolation was found in both groups and, depending upon the individual, had either a diffuse or multifocal distribution. Similarly, there was also variability in the relative abundance of melanin macrophage centers in the liver.

One change was noted solely in the experimental animals. Five of the nine exposed fish were found to have extravasated blood either in the peritoneal or pericardial cavity, the extent of which varied from mild to severe (Figure 1).

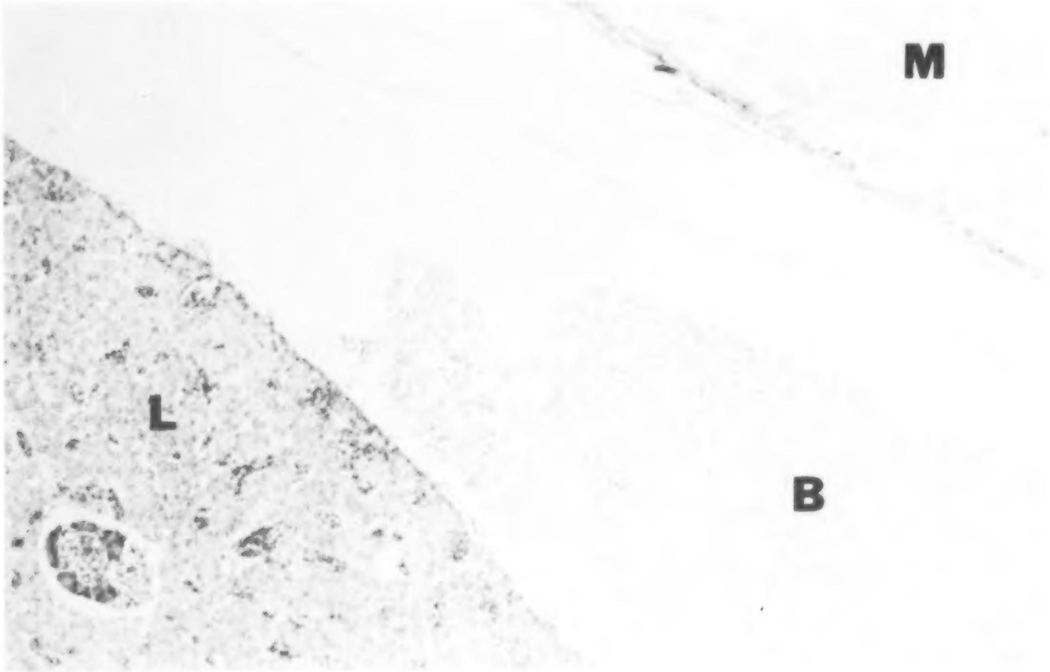

Figure 1. Extravasation of blood (B) into the peritoneal cavity. Liver (L), body wall musculature (M). Hematoxylin and eosin, 80 x.

Hematology

At the termination of the test, blood was drawn from 15 fish (11 experimental, 4 control) for determination of hematocrit levels and for differential blood cell counts.

The hematocrit values were quite low compared with those published for other fish species, but were fairly consistent for both exposed and unexposed fish (Table 4). The experimental fish did have a slightly lower mean value of 12.7% compared to 16.2% for the controls.

TABLE 4. PACKED RED BLOOD CELL VOLUMES (HEMATOCRIT) OF JUVENILE ENGLISH SOLE EXPOSED AND UNEXPOSED TO WATERBORNE BaP FOR 30 DAYS

Group	Specimen Number		Hematocrit (%)
I. Control	24		18
	22		13
	19		16
	20		18
		MEAN	16.2
II. Experimental	13		7
	15		11
	16		12
	18		10
	17		20
	27		13
	21		12
	14		13
	25		3
	26		16
	28		23
		MEAN	12.7

Leucocyte differentials were classified according to Ellis (1976) and revealed a disparity between the experimental and control fish (Table 5). Because of poor fixation, it was not possible to reliably distinguish lymphocytes from thrombocytes, which were therefore scored together.

In the control fish, the predominant cell type was the small lymphocyte/thrombocyte. These cells accounted for an average of 66% of the white cells. In experimental fish, these cells were also the most predominant but represented an average of only 40%. In the control fish, granulocytes accounted for 26% of the white blood cells and in the experimental fish, 38%. The most striking difference between the two groups was in the number of very large cells which appeared to represent immature blast cells and, to a lesser extent, monocytes. In the control fish, the large cells made up an average of 8% of the cells, but in the experimental animals this value almost tripled to an average of 22%. Without special stains the identity of the blast forms could not be determined; however, they were most compatible with cell types from either the erythrocytic or granulocytic series.

TABLE 5. LEUCOCYTE DIFFERENTIAL COUNTS FOR JUVENILE ENGLISH SOLE EXPOSED AND UNEXPOSED TO WATERBORNE BaP FOR 30 DAYS

Group	Specimen Number	Lymphocytes/ thrombocytes (%)	Granulocytes (%)	Monocytes/ blast cells (%)
Control	24	66	32	2
	22	82	14	4
	19	68	18	14
	20	52	38	10
	MEAN	66	26	8
Experimental	13	24	28	48
	15	16	52	32
	16	14	40	46
	18	36	36	28
	27	48	48	4
	14	60	38	2
	25	20	46	28
	26	68	22	10
	28	72	26	6
	MEAN	40	38	22

DISCUSSION

Toxicity tests involving heterogeneous populations of wild fish present a formidable challenge which should not be undertaken lightly. Unlike the laboratory mouse, fish collected in the field represent a diverse group of individuals with varied genetic, nutritional, and health histories. In light of this inherent diversity, test conditions should be as meticulous as possible so that data can be interpreted reasonably. This care is particularly critical when low levels of toxicants are used.

To this end, the fish used in our study were collected from a site removed from industrial activity. The fish were maintained in artificial seawater free of petroleum hydrocarbons and were housed in an aquarium system constructed entirely of glass and teflon to avoid leaching of extraneous compounds associated with certain plastics, PCV tubing, and metals. The use of control animals housed under similar conditions does not lessen the need for such precautions since a synergistic effect may be utterly different from that caused by the compound under study. One further protective measure was purification of the test compound. Such processing revealed to us the presence of a polar contaminant which behaved as a direct mutagen (unpublished data) in the Ames *Salmonella* assay (Ames *et al*., 1975), and which could have altered the results of our experiment.

Quantitation of BaP in Seawater

Studies have been conducted exposing fish and shellfish to waterborne BaP (Lee *et al*., 1972a, 1972b; Lee, 1975; Neff and Anderson, 1975; Lee *et al*., 1976). In some of these studies, the actual concentration of the compound in solution was not distinguished from that in crystalline form. Tests in our laboratory indicate that BaP is rapidly removed from solution and that large portions of the compound are not readily available to fish. Investigators should note this fact when reporting exposure conditions.

Uptake of BaP

Studies of whole-body accumulation of waterborne BaP (Lee *et al*., 1972b) have frequently either failed to consider surface adsorption of the chemical or have attempted to remove adhering particles by aqueous washings. Due to the non-polar nature of BaP, such procedures are unsatisfactory. The substitution of organic solvents, such as methylene chloride, provides more complete removal. Distinction between adherent particles and tissue concentrations are critical to accurate determinations of body burdens.

Our study did not attempt to distinguish metabolites; however, further tests in our laboratory (unpublished data), in which metabolites were identified, verified that substantial quantities of BaP are stored as parent compound. The fish used in the current experiment exhibited marked variability in bioaccumulation of BaP. This may have reflected differential ability to metabolize the substance.

Hepatic AHH Activity

Measurement of piscine microsomal enzyme activities has been suggested as a means of monitoring aquatic pollution (Payne, 1976). Results from our study suggest that AHH activity may be a poor index of chemical exposure.

In the fish examined following 30-day exposure, two distinct groups of organisms emerged. Enzyme levels in one group were equal to or lower than those of control fish; but dramatically increased in the other. This disparity might be explained by genetic factors that render the animal either responsive or nonresponsive to induction for a given enzyme system. Such a phenomenon is known in man for AHH (Conney and Burns, 1972). If piscine familial differences exist in terms of enzyme activity, they might help to identify sub-populations of high-risk animals.

Histopathology and Hematology

The presence of extravasated blood in 55% of the test animals is suggestive either of endothelial damage or of alterations in membrane permeability. The possibility cannot be excluded, however, that bleeding may have resulted from trauma, such as that induced by migrating parasites. Further light and electron microscopy will be needed to resolve the question; however, similar results have been noted in other fish species exposed to petroleum hydrocarbons (Vishnevetskii, 1961).

The shift toward more immature white blood cells in peripheral blood may have resulted from alterations in hematopoietic tissue. Even though no lesions were noted in the anterior kidney, results from other waterborne and injection experiments that we conducted corroborate these findings. The implication of such damage is obvious. If leucopoiesis were impaired, the animal would have decreased resistance to infectious agents and might be more susceptible to neoplasia due to diminished cell mediated immunity (Rubin, 1964).

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ACCUMULATION AND RELEASE OF POLYCYCLIC AROMATIC HYDROCARBONS FROM WATER, FOOD, AND SEDIMENT BY MARINE ANIMALS

by

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ABSTRACT

All species of marine organisms studied to date rapidly accumulated polycyclic aromatic hydrocarbons (PAH) from low concentrations in the ambient water. Bioaccumulation factors tend to increase as the molecular weight of the PAH increases. The patterns of PAH accumulation from dispersed or water-soluble fractions of oil are complex and variable. PAHs adsorbed to food are accumulated only to a very limited extent by marine polychaete worms and fish. However, accumulation of PAH from food is many times more efficient than accumulation from water by some marine crustaceans. The bioavailability to benthic marine animals of sediment-adsorbed PAH is very limited. Animals collected from PAH-contaminated sediments generally have lower concentrations of PAH in their tissues than the PAH concentration in the sediment. When returned to a PAH-free environment all marine animals studied to date rapidly released PAH from their tissues to low or undetectable levels. PAH metabolism is important but not essential for PAH release. Many endogenous and exogenous factors affect the rates of PAH uptake and release by marine organisms.

INTRODUCTION

The presence of polycyclic aromatic hydrocarbons (PAHs) in tissues of a wide variety of marine organisms (Neff, 1978) strongly indicates that these organisms are able to accumulate PAH present at low concentrations in the ambient medium, food, or sediments. This is not surprising, since PAHs are highly hydrophobic and lipophilic. Intrinisic lipid/water partition coefficients favor their rapid transfer from the aqueous phase into lipophilic compartments such as biological membranes, macromolecules, and depot lipid stores in organisms (Leo et al., 1971; Neely et al., 1974).

Release of PAH from tissues of contaminated organisms may be passive, reflecting an equilibrium distribution between the aqueous phase and lipophilic compartments in contract with it, or it may be active and involve metabolic transformation of PAH to polar water-soluble metabolites which are more readily excreted. A large amount of research has been conducted in recent years on accumulation and release of petroleum hydrocarbons by marine organisms (see reviews of Anderson et al., 1974a; Varansi and Malins, 1977). Only literature dealing specifically with accumulation and release of PAH by marine organisms will be reviewed.

Accumulation and Release of PAH from Water

Polychaete worms, *Neanthes arenaceodentata*, exposed for 24 hr to seawater containing 0.15 parts per million (ppm) ^{14}C-naphthalene, accumulated naphthalene to a maximum of 6 μg/g tissue (ppm) in 3 to 24 hr (Rossi, 1977). On return to isotope-free seawater, ^{14}C-naphthalene was rapidly released and reduced to nondetectable levels within 300 hr. Approximately one-third of the radioactivity released by *Neanthes* during the first 24 hr of depuration was in the form of unmetabolized naphthalene. The remainder was in the form of polar metabolites. Significant levels of ^{14}C-naphthalene metabolites remained in tissue material after 504 hr (21 days) of depuration, suggesting that some metabolic products were covalently bound to tissue macromolecules.

Several studies have been performed on accumulation and release of PAH in solution by marine bivalve molluscs. Lee et al. (1972a) demonstrated accumulation by the mussel, *Mytilus edulis*, of ^{14}C-naphthalene and ^{3}H-benzo(a)pyrene dissolved in seawater. The highest levels of activity were recorded in the gill; the authors hypothesized that gill tissue of mussels has a micellar layer which absorbs hydrocarbons and then passes them to other tissues. Although no evidence of MFO activity could be detected, mussels rapidly released accumulated PAH when returned to isotope-free seawater.

Neff et al. (1976a) measured relative rates of accumulation and release of 4 PAHs from seawater by the estuarine clam, *Rangia cuneata*, (Table 1). Phenanthrene was accumulated most rapidly and released most slowly. The rapid release of naphthalene from clam tissues probably masked a similarly rapid uptake during exposure, since both influx and efflux of this compound undoubtedly occurred simultaneously. These results can best be explained in terms of relative aqueous solubilities and lipid/water partition coefficients of the four PAHs. Naphthalene is the most water-soluble of PAHs tested and thus more readily bioavailable. Although it has a high affinity for lipids, its lipid/water partition coefficient favors rapid release to water when naphthalene concentrations in the medium are reduced. Benzo(a)pyrene (BaP), at the other extreme, has a very low aqueous solubility (1 ppb) so that most BaP in the exposure water was in colloidal or particulate form, decreasing its bioavailability.

Once absorbed, BaP would not readily partition back into the aqueous phase even when concentrations in the medium were extremely low. In other experiments *R. cuneata* accumulated up to 7.2 ppm BaP during 24-hr exposure to 0.03 ppm ^{14}C-BaP in seawater (Neff and Anderson, 1975). Nearly 75% of the activity was located in the viscera (digestive gland, gonad, etc.). When returned to isotope-free seawater, clams released BaP to undetectable levels in 58 days. Tissue distribution of BaP remained relatively constant during the depuration period. Dunn and Stich (1976) measured the rate of release of BaP from *Mytilus edulis* naturally contaminated with PAH. Concentration of BaP in mussel tissues was approximately 45 μg/kg wet weight at the beginning of the experiment. When placed in clean seawater, mussels released BaP at an approximately exponential rate over the six-week depuration period. The overall half-life of BaP in mussel tissues was approximately 16 days.

TABLE 1. ACCUMULATION FROM SEAWATER AND RELEASE OF PAH BY THE ESTUARINE CLAM, *RANGIA CUNEATA* (FROM NEFF *et al*., 1976A)

PAH	Naphthalene	Phenanthrene	Chrysene	Benzo(a)pyrene
Exposure Concentration (ppm)	0.071	0.089	0.066	0.052
Tissue concentration after 24 hr exposure (ppm) ± S.D.	0.43±0.1	2.85±1.1	0.54±0.3	0.45±0.1
Bioaccumulation factor [tissue]/[water]	6.1	32.0	8.2	8.7
Tissue concentration after 24 hr depuration (ppm) ± S.D.	0.15±0.02	2.47±1.2	0.40±0.15	0.38*
% Released in 24 hr	66	13	26	16

* only one sample analyzed.

Lee *et al*. (1978) suspended oysters, *Crassostrea virginica*, at 7-m depth in a controlled ecosystem enclosure, which was dosed with Prudoe Bay crude oil enriched with several PAHs. Some oysters were subsequently removed to hydrocarbon-free seawater for depuration studies.

Naphthalene and alkylnaphthalenes were accumulated most rapidly by the oysters (Table 2). Rate of accumulation of other PAHs decreased with increasing molecular weight. When oysters were returned to clean seawater, naphthalenes were released rapidly and reached undetectable levels in 23 days. Anthracene, fluoranthene, benz(a)anthracene, and BaP were released much more slowly. Based on the depuration experiments, calculated half-lives of the naphthalenes, anthracene, fluoranthene, benz(a)anthracene, and BaP were 2, 3, 5, 9, and 18 days, respectively. Thus, despite the very limited ability of molluscs to metabolize PAH, all species so far studied are able to release the majority of accumulated PAH from their tissues in periods varying from a few days to several weeks.

Patterns of accumulation and release of PAHs in solution by aquatic crustaceans are somewhat different, probably reflecting their more active mode of life and greater PAH-metabolizing abilities. The copepod, Calanus helgolandicus, was able to accumulate naphthalene during exposure for 24 hr to seawater solutions containing as little as 0.1 μg naphthalene/ℓ (ppb) (Corner et al., 1976a). When returned to naphthalene-free seawater, copepods released naphthalene rapidly (half-life ∿1.5 days). Depuration was slightly more rapid in copepods feeding on algal cells than in starved individuals. Subsequently, Harris et al. (1977b) studied accumulation of ^{14}C-naphthalene by C. helgolandicus and Eurytemora affinis during exposure to concentrations of 0.2 to 992 μg ^{14}C-naphthalene/ℓ seawater for up to 15 days. Initial uptake was rapid but after exposure for seven to eight days to seawater containing 50 μg/ℓ an equilibrium condition was approached. At an exposure concentration of 1 μg ^{14}C-naphthalene/ℓ, the quantity of radioactivity accumulated in 10 days was nearly 50 times greater in the smaller estuarine species, E. affinis, than in the larger oceanic species, C. helgolandicus, when expressed in terms of body weight. The difference in uptake rate was not due to differences in lipid content between the two species. When returned to hydrocarbon-free seawater, C. hegolandicus released up to 90% of the accumulated radioactivity in 24 hr. However, 5% of the accumulated radioactivity remained in the copepods after 11 days. Much of the radioactivity in the copepods and depuration water was identified as naphthalene metabolites.

Lee (1975) obtained similar results when he exposed several species of marine zooplankton to seawater solutions of radio-labeled BaP, methychol-anthrene, and naphthalene. The copepod, Calanus plumchrus, accumulated up to 22 x 10^{-4} μg BaP/individual during a three-day exposure to 1 μg BaP/ℓ in seawater (Figure 1). When returned to isotope-free seawater, the copepod released most of the accumulated radioactivity in 17 days. When depuration was continued beyond 17 days, no further hydrocarbon loss was observed. PAH metabolism contributed significantly to release of PAHs by all crustaceans studied.

Pink shrimp, Penaeus duorarum, exposed to 1 or 5 μg chrysene/ℓ seawater accumulated the PAH in both the cephalothorax and abdomen (Miller et al., 1978). At an exposure concentration of 5 ppb, shrimp accumulated approximately 1.8 μg chrysene/g tissue in the cephalothorax and 0.4 μg chrysene/g tissue in the abdomen in 28 days. When returned to clean

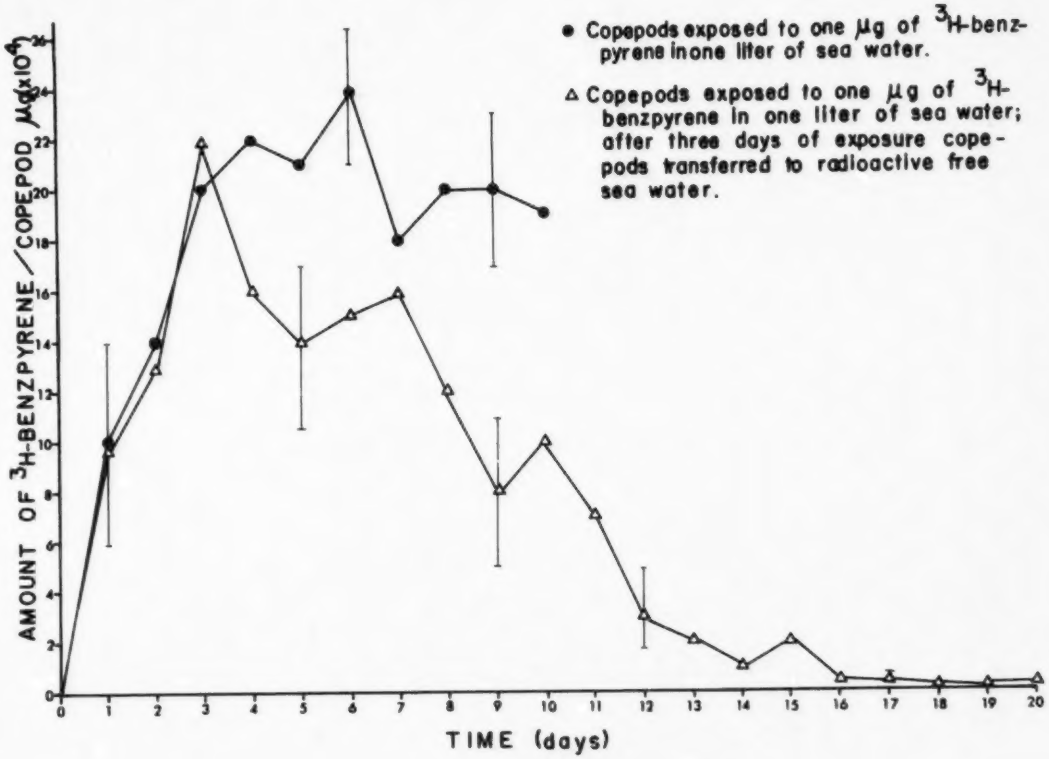

Figure 1. Accumulation of benzo(a)pyrene by the copepod Calanus plumchrus during exposure to 1 μg BaP/ℓ seawater and BaP release following return to clean seawater (From Lee, 1975).

TABLE 2. ACCUMULATION AND RELEASE OF PAH BY THE OYSTER, *CROSSOSTREA VIRGINICA*, EXPOSED TO CRUDE OIL TREATED WITH SEVERAL PAHs IN A CONTROLLED ECOSYSTEM ENCLOSURE (FROM LEE *ET AL*., 1978)

Duration of Exposure (days)	Duration of Depuration (days)	Naphthalene		Methylnaphthalenes		Dimethylnaphthalenes	
		Oyster (μg/g)	Water (μg/ℓ)	Oyster (μg/g)	Water (μg/ℓ)	Oyster (μg/g)	Water (μg/ℓ)
2		30	5	58	8	84	10
8		12	3	36	3	72	2
2	7	1	-	1	-	8	-
8	7	2	-	2	-	4	-
8	23	N.D.*	-	N.D.	-	N.D.	-

Duration of Exposure (days)	Duration of Depuartion (days)	Anthracene		Fluoranthene		Benz[a]anthracene		Benzo[a]pyrene	
		Oyster (μg/g)	Water (μg/ℓ)	Oyster (μg/g)	Water (μg/ℓ)	Oyster (μg/g)	Water (μg/ℓ)	Oyster (μg/g)	Water (μg/ℓ)
2		5.6	13	5.0	7.2	2.8	5.3	0.36	1.9
8		2.5	1	4.0	0.4	1.8	0.1	0.30	0.1
2	7	1.2	-	1.7	-	1.9	-	0.40	-
8	7	0.4	-	1.4	-	1.0	-	0.20	-
8	23	0.1	-	0.4	-	0.3	-	0.12	-

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* N.D.=not detected, less than 0.5 μg/g.

seawater, shrimp released most of the chrysene in 10 days. However, a small but measurable amount of chrysene remained in their tissues after 28 days of depuration. No attempt was made to measure metabolite formation.

Juvenile blue crabs, *Callinectes sapidus*, accumulated isotopically labeled BaP, methylcholanthrene, and fluorene during exposure to seawater containing 2.5, 1.0, and 30 µg/ℓ of these compounds, respectively (Lee *et al*., 1976). Maximum radioactivity in the crabs was reached after two days, although uptake from the water continued beyond this time. After a two-day exposure, rapid discharge of PAHs and metabolites from the crabs balanced uptake from the medium. Initial uptake took place via gill tissue, from which radioactivity was transferred primarily to the blood and hepatopancreas. The pattern of accumulation, distribution in the body, and metabolism and release of ^{3}H-BaP by crabs is summarized in Table 3. Main sites of BaP accumulation were the hepatopancreas and gill. During both the exposure and depuration periods the fraction of total radioactivity present as unmetabolized BaP decreased with time in all tissues analyzed. At every sampling period, more than 50% of accumulated radioactivity was in the hepatopancreas and, in all but the Day 1 samples, more than 50% hepatopancreatic activity was present as hydrocarbon metabolites. During depuration, concentrations of BaP and its metabolites in the crab tissues dropped rapidly, so that after 20 days only small amounts of radioactivity remained, primarily in the hepatopancreas as polar metabolites. BaP and metabolites were recovered from depuration water.

Throughout the first four days of depuration, the major excretory product was unmetabolized BaP. After longer depuration times, major excretory products were various polar metabolites. More than 50% accumulated BaP, and its metabolites were excreted in six days. More than 70% radioactivity excreted was in particulate form, suggesting that fecal material was the main route of PAH excretion. A fraction of the total radioactivity in crab tissues (varying from 0.3 to 50% of the total) occurred in a form not extractable with the solvents used. This material may have consisted of highly polar compounds or BaP metabolites that were covalently bound to tissue macromolecules. The relative proportion of this non-extractable radioactivity in the tissues increased during the depuration period, implying that it was not as readily excreted as BaP or its major metabolites. Similar conclusions were obtained with fluorene and methylcholanthrene. All results dramatically demonstrate the importance of PAH metabolism in elimination of these materials from tissues of PAH-contaminated crustaceans.

The observations reported in several of the studies discussed above suggest that some of the products of PAH metabolism may be retained in animal tissues longer than unmetabolized PAH. A recent study by Sanborn and Malins (1977) supports this view. Stage V spot shrimp larvae, *Pandalus platyceros*, accumulated high concentrations of radioactivity during exposure to 8-12 µg ^{14}C-naphthalene/ℓ in water. Metabolic products of naphthalene accounted for up to 21% of the radioactivity in larval tissues. ^{14}C-naphthalene was almost completely depurated from the tissues during 24 to 36 hr in isotope-free seawater. However, metabolic products were strongly resistant to depuration.

TABLE 3. FATE OF ^{3}H-BENZO(a)PYRENE ACCUMULATED FROM WATER (2.5 μg/ℓ BaP) BY JUVENILE CRABS, _CALLINECTES SAPIDUS_. AFTER TWO-DAY EXPOSURE, CRABS WERE TRANSFERRED TO HYDROCARBON-FREE SEAWATER FOR DEPURATION. VALUES ARE MEANS FOR 3 CRABS SEPARATELY ANALYZED ±1 S. D. (LEE _et al_., 1976)

Product	Time (days)	Quantity in tissue (μg) x 10^4				
		Gill	Blood	Hepato-pancreas	Stomach	Muscle
Total	1	150±70	90±17	270±90	14±2	10±3
	2	410±85	85±20	590±210	11±6	18±7
	4	100±32	80±52	300±18	10±4	8±5
	8	70±55	65±21	210±16	4±5	4±3
	12	35±12	20±13	320±74	6±2	2±2
	20	1±2	6±5	40±26	2±1	2±3
Benzo(a)pyrene	1	110	50	160	3	4
	2	290	30	280	2	2
	4	70	12	40	1	2
	8	22	8	21	t*	1
	12	10	1	32	t	t
	20	t	1	2	t	t
Hydroxybenzo(a)pyrene	1	10	27	20	2	2
	2	15	12	84	2	2
	4	5	21	90	2	1
	8	11	9	40	t	t
	12	7	6	24	1	t
	20	t	2	5	t	t
Polar metabolites	1	15	10	89	8	2
	2	3	26	210	6	8
	4	12	32	140	7	4
	8	17	36	130	2	2
	12	11	10	224	2	1
	20	t	2	29	1	1

*t=detectable but less than 0.5 x 10^{-4} μg.

Most fish are able to metabolize and excrete PAH accumulated from the medium even more rapidly than crustaceans. Anderson et al. (1974b) reported that accumulation and release of naphthalene and 1-methylnaphthalene were very rapid in the estuarine sheepshead minnow, Cyprinodon variegatus. When exposed for 4 hr to 1 ppm of each compound in seawater the fish accumulated 60 ppm naphthalene and 210 ppm 1-methylnaphthalene. Nearly 90% of the accumulated hydrocarbons were released after 29 hr in hydrocarbon-free seawater.

Lee et al. (1972b) studied accumulation and release of ^{14}C-naphthalene and ^{3}H-BaP by three species of marine fish. All three species rapidly accumulated the PAH from diluted solution in seawater. Equilibrium levels in the tissues were reached in about 1 hr. The main route of uptake appeared to be through the gills. Both naphthalene and BaP tended to accumulate primarily in the liver and gallbladder; the latter organ contained the majority of the activity after several hours depuration. Following 24 hr in clean seawater, more than 90% of radioactivity accumulated as ^{14}C-naphthalene was lost from most of the fish tissues. BaP was released more slowly with losses of 50, 50, 90, and 20% radioactivity in liver, gut, gill, and flesh, respectively, after 24 hr in clean seawater. A significant portion of the radioactivity excreted following exposure of fish to both ^{14}C-naphthalene and ^{3}H-BaP was in the form of polar metabolites. The main routes of excretion of PAHs and their metabolites were the gallbladder and urine.

Statham et al. (1976) showed that the gallbladder was a principal site of accumulation of several hydrophobic xenobiotics in trout, Salmo gairdneri. However, the highest concentrations of radioactivity detected in mummichogs, Fundulus heteroclitus, following exposure for 4 hr to ^{14}C-naphthalene was in the spleen (34 to 105 times the exposure concentration)(DiMichele and Taylor, 1978). Liver, brain, anterior kidney, and gallbladder also contained high concentrations of radioactivity. No attempt was made to identify metabolites. On the other hand, mangrove snapper, Lutjanus griseus, exposed to 1 or 5 μg chrysene/ℓ seawater, accumulated PAH only in the liver and not in other tissues examined (gallbladder, white muscle, intestine)(Miller et al., 1978). These disparate results suggest either that there are interspecific differences in distribution in fish tissues of PAH accumulated from water or that different PAHs have different distribution patterns in the tissues.

The role of the MFO system in excretion of PAH by rainbow trout, Salmo gairdneri, was investigated by Statham et al. (1978). Trout that were pretreated (induced) with benzanthracene and exposed to ^{14}C-2-methylnaphthalene in solution, exhibited significantly elevated rates of bilary excretion of accumulated ^{14}C-2-methylnaphthalene and metabolites. The bile of induced fish contained a higher proportion of polar metabolites of 2-methylnaphthalene than that of controls. Initial levels of ^{14}C were higher in livers of induced trout than in uninduced controls, and radioactivity appeared to be retained longer in livers of induced fish during the depuration period. Pretreatment with benzanthracene had little effect on the rate of disappearance of radioactivity from blood and muscle. The greater retention of ^{14}C in livers of induced fish may be due to the metabolic production of electrophylic metabolites which became bound to tissue macromolecules.

Sharp et al. (1978) showed that rates of uptake and release of ^{14}C-naphthalene changed during the timecourse of embryonic development of the mummichog, Fundulus heteroclitus. The uptake rate of naphthalene decreased markedly from 1265 DPM ^{14}C/embryo/hr on day two of development to 195 DPM ^{14}C/embryo/hr on Day 10 of development. Water influx rates varied only slightly during the same time period. Rate of ^{14}C-naphthalene efflux from the embryos, when returned to isotope-free seawater, varied from 8.65 to 9.95%/hr between Day 2 and Day 6 of development and then dropped to 5.85%/hr on Day 12 of development. Embryos were unable to metabolize either naphthalene of chrysene to polar metabolites at any stage of embryonic development, possibly accounting for the relatively slow depuration rate.

Accumulation of PAH from Food

Considerably less research has been done on the ability of aquatic animals to accumulate PAH from food sources. Such information is, of course, necessary to assess the potential for biomagnification of PAH in aquatic food chains.

Rossi (1977) fed young adult polychaete worms, Neanthes arenaceodentata, powdered alfalfa (their normal diet in laboratory culture) contaminated with 10 to 15 ppm ^{14}C-2-methylnaphthalene for 16 days in succession. Radioactivity was detected in the worms after feeding for 192 and 384 hr on contaminated food. If worms were given uncontaminated fish food for 24 hr to allow purging of labeled unassimilated food, no radioactivity was detected in tissue material at any sampling time. In addition more than 85% of radioactivity recovered from the water of the exposure chambers occurred as the unmetabolized parent compound. Thus, N. arenaceodentata had little if any ability to accumulate 2-methylnaphthalene from its food.

The situation is quite different in marine crustaceans and fish. Corner et al, (1976b) studied dietary uptake of ^{14}C-naphthalene by the marine copepod, Calanus helgolandicus. When Calanus were fed ^{14}C-naphthalene-contaminated living or dead copepod nauplii, Elminius sp., or living algae, Biddulphia, ^{14}C-naphthalene was accumulated. Uptake from food was much more efficient than uptake from solution. Approximately 35% of radioactivity taken from the food source was lost during 24-hr depuration, after which 94% of that remaining in the copepods could still be identified as unmetabolized hydrocarbon. However, less than one-third of the radioactivity in depuration water existed as unmetabolized naphthalene. Similar results were obtained by Harris et al. (1977a), who compared ^{14}C-naphthalene uptake from solution versus food supply (Biddulphia sinensis cells). Compared to the quantity of ^{14}C-naphthalene present as suspended food, the amount in solution alone required to give the same increase in hydrocarbon level in copepods was several orders of magnitude greater in experiments with female *Calanus* and two orders of magnitude greater with males (Table 4). The discrepancy between male and female responses was attributed to the higher feeding rate of females in comparison to the smaller males. Approximately 60% of naphthalene ingested with the food was assimilated by both male and female copepods (Table 5). Of the assimilated naphthalene, almost half was retained in copepod tissues; the other half was released as either unmetabolized naphthalene or its metabolites.

TABLE 4. QUANTITATIVE IMPORTANCE OF THE DIETARY PATHWAY IN THE ACCUMULATION OF ^{14}C-NAPHTHALENE BY THE MARINE COPEPOD, *CALANUS HELGOLANDICUS* (FROM HARRIS *et al*., 1977A)

Hydrocarbon concentration		Radioactivity as pg hydrocarbon/animal		Hydrocarbon in soln. alone equivalent to (soln & food) level (µg/ℓ)	Ratio (C-A:B)
In soln (µg/ℓ) A	As food (ng/ℓ) B	Soln alone	Soln & Food	C	
Experiments with females					
0.96	0.66	53	106	2.40	2186
4.69	2.38	179	386	11.55	2892
25.52	122.40	759	4538	230.80	1677
26.00	29.00	749	1830	76.54	1743
134.10	61.40	2997	6774	375.40	3930
Experiments with males					
0.93	1.10	75	88	1.24	282

TABLE 5. FATE OF ^{14}C-NAPHTHALENE ACCUMULATED BY *CALANUS HELGOLANCICUS* FROM INGESTION OF CONTAMINATED *BIDDULPHIA* CELLS (FROM HARRIS et al., 1977A)

	% Ration				Retained	Soluble release
	Feces	Assimilated	Retained	Soluble release	Assimilated %	Assimilated %
Female	41.9	58.1	31.2	26.9	53.7	46.3
Male	39.3	60.7	26.8	33.9	44.1	55.9

Lee *et al*. (1976) showed that juvenile blue crabs, *Callinectes sapidus*, were able to accumulate naphthalene, methylnaphthalene, fluorene, and BaP from food. When crabs were fed shrimp or oysters containing radiolabeled PAH, between 7 and 10% of the radioactivity was transferred from the stomach to other body tissues. Most of the remainder was unabsorbed and lost in the feces during the first two days after feeding. Radioactivity appeared in the hepatopancreas after 6 hr and in the blood after 18 hr. Gills, muscles, and gonads also contained significant levels of radioactivity. The pattern of PAH release form the tissues following ingestion of contaminated food was qualitatively similar to that described earlier for depuration of PAH accumulated from water. A significant fraction of radioactivity was released as polar metabolites.

Dixit and Anderson (1977) administered ^{14}C-naphthalene to Gulf killifish, *Fundulus similis*, by stomach tube. After 2 hr, 34% of the administered radioactivity was recovered in tissue material, 34% of this was in stomach tissue. Thus, approximately 12% of the administered ^{14}C-naphthalene was assimilated by *Fundulus*. The majority of this activity was localized in the liver and gallbladder after 2 hr. Significant amount of activity were also present in the heart and lateral body musculature. After 8 hr, 79% of the radioactivity recovered was present in the gallbladder. These results strongly suggest that PAHs absorbed from the gut are transported to the liver where they are rapidly metabolized and excreted in bile.

Roughly similar results were obtained when ^{14}C-BaP contaminated squid were fed to young cod, *Gadus morrhua*, (Corner *et al*., 1976b) or juvenile herring, *Clupea harengus* (Whittle *et el*., 1977); 48 hr after feeding, 83.5% of the radioactivity was still present in the stomach and 12% in intestinal contents of the cod (Table 6). The liver, bile fluid, and gills were the only other tissues containing significant activity. After 72 hr the bile fluid contained 12.5% of the total activity and after 96 hr the intestinal contents and feces contained 57% of the total recovered radioactivity; most of the remainder (37.1%) was associated with the stomach. In herring, nearly 80% of the recovered ^{14}C remained in the lipid fraction (unmetabolized BaP) of the stomach 43 hr after ingestion of ^{14}C-BaP contaminated squid. The largest fraction of the remaining activity was found in the lipid fraction of the intestine (10.3%) and the residual fraction (unextractable BaP metabolites) of the stomach, pyloric caecae, and intestine (4.9%). Most of the activity recoverd in bile was water-soluble, indicating polar metabolites. Although the digestive tract of fish represents the major site of both uptake and excretion of orally administered PAH, the fact that more than 98% of radioactivity recovered from the fish 43 hr after feeding was in the digestive tract strongly suggests that there was very little assimilation of ingested BaP, and that the small amount of BaP that was assimilated was rapidly metabolized and excreted via the gallbladder into the intestine. The authors concluded that retention of BaP in the stomach implies strong adsorption or binding to the stomach wall. This binding prevents subsequent absorption an assimilation of ingested BaP.

TABLE 6. DISTRIBUTION OF ^{14}C ACTIVITY (AS % OF TOTAL ACTIVITY RECOVERED AT INTERVALS AFTER FEEDING SQUID CONTAINING ^{14}C-BaP AND ^{14}C-HEXADECANE TO YOUNG CODFISH, GADUS MORRHUA (FROM CORNER et al., 1976B)

Sample	% ^{14}C activity recovered after		
	48 hr	72 hr	96 hr
Stomach	83.5	32.9	37.1
Liver	2.2	3.6	3.4
Bile fluid	0.6	12.5	1.9
Intestinal contents	12.9	8.6*	36.3
Urine	0	trace	0.03
Aquarium residue (mainly feces)	0.5	41.4	20.7
Aquarium water	0	0.2	0.12
Plasma**	0.03	0.08	0.05
Blood**	0	0.06	0.04
Gills	0.2	0.5	0.4
Spleen	0.06	0.1	0.1

*A loss of at least 50% occurred during dissection.
**Expressed on a per gram basis.

Twenty-four hours after fingerling coho salmon, Oncorhynchus kisutch, were fed food containing ^{14}C-naphthalene or ^{14}C-anthracene, 0.03 and 0.17%, respectively, of the administered radioactivity was associated with the liver, brain, and flesh of the fish (Roubal et al., 1977b). The specific activity in the liver and brain was higher than in flesh, but the flesh contained more total activity. Anthracene and its metabolites were retained longer in fish tissues than were naphthalene and its metabolites.

From the limited data available, it would appear that there are large interspecific differences in ability to absorb and assimilate PAH from food. Polychaete worms have a very limited ability to absorb and assimilate PAH, whereas fish adsorption of PAH from the gut is limited and variable depending on species of fish, the PAH, and possibly the food matrix in which PAH is administered. Crustaceans, on the other hand, apparently readily assimilate PAH from contaminated food. In all cases where assimilation of ingested PAH was demonstrated, metabolism and excretion of PAH were rapid. Thus, the potential for food chain biomagnification of PAH seems to be limited. For such biomagnification to occur, the material must be readily absorbed from food, and once assimilated, it must be relatively resistant to metabolism or excretion.

Accumulation of PAH from Petroleum

When petroleum is spilled in water, PAHs in the oil may enter the water column as PAH in solution, in dispersed form (micro- or macro-oil droplets), or adsorbed to organic or inorganic particulates. The physical form in which oil-derived PAHs occur in the water column may significantly affect the rate at which they are accumulated by aquatic organisms. Several investigators have studied accumulation of petroleum in different aqueous forms.

As indicated above, when aquatic animals are exposed to a single PAH or a simple mixture of the PAH in solution, there is usually a good correlation between octanol/water partition coefficient of each PAH and its bioaccumulation factor (concentration in tissue/concentration in water) (Neeley *et al*., 1975; Neff *et al*., 1976a; Lu *et al*., 1977). Therefore, biomagnification factors increase as molecular weight of the PAH increases. Based on these considerations, one might predict that the aromatic fraction in tissues of oil-contaminated marine animals would be enriched in higher molecular weight PAH as compared to the aromatic fraction of the contaminating oil. This is not the case when short-term exposure to dispersed oil occurs. Oysters, *Crassostrea virginica*, subjected for 8 hr to a concentrated oil-in-water dispersion of No. 2 fuel oil (302 ppm total hydrocarbons at 8 hr), accumulated a wide spectrum of hydrocarbons (Table 7)(Neff *et al*., 1976b). By the end of the exposure period, oysters had accumulated a total of 311 ppm total hydrocarbons, including 76.7 ppm PAH. The relative concentrations of different PAHs in the oyster tissues were similar to those in the oil; there was no evidence of selective accumulation of higher or lower molecular weight PAH. When returned to oil-free seawater, the oysters released n-paraffins rapidly; PAHs were released more slowly. All PAHs showed roughly similar behavior. Concentrations of different PAHs remained essentially constant during the first 120-hr post-exposure. However, after 672 hr (28 days), the concentration of PAH in oyster tissues reached background levels. Clams, *Rangia cuneata*, exposed to the same oil dispersion accumulated only 89.6 ppm total hydrocarbons in 8 hr (28.5% of the amount accumulated by oysters in the same time period). The pattern of petroleum hydrocarbon accumulation and release by the clams was qualitatively similar to that of the oysters.

Bieri and Stamoudis (1977) obtained similar results when they exposed oysters, *Crassostrea virginica*, and hard shell clams, *Mercenaria mercenaria* to an experimental spill of No. 2 fuel oil. The oysters rapidly accumulated aliphatic and aromatic hydrocarbons after the spill. Between 6 and 25 hr after the spill, most of the n-alkanes had been released from oyster tissues. Branched alkanes and olefins were released more slowly. Patterns of PAH accumulation and release were more complex (Table 8). The concentrations in oyster tissues of alkyl naphthalens with up to five alkyl carbons, biphenyls with up to two alkyl carbons, and fluorenes with up to one methyl group increased until 25 hr after the spill. Maximum tissue

TABLE 7. ACCUMULATION OF PETROLEUM HYDROCARBONS BY OYSTERS *CRASSOSTREA VIRGINICA* DURING EXPOSURE TO DISPERSED NO. 2 FUEL OIL IN A FLOW THROUGH SYSTEM AND SUBSEQUENT RELEASE OF HYDROCARBONS WHEN OYSTERS WERE RETURNED TO OIL-FREE SEAWATER. THE COMPOSITION OF THE NO. 2 FUEL OIL IS INCLUDED FOR COMPARISON (FROM ANDERSON *et al.*, 1974B; NEFF *et al.*, 1976B)

Time (hr)	Petroleum hydrocarbon concentration (ppm, μg/g wet wt)*														
	n-P	N	1-MN	2-MN	DMN	TMN	B	MB	F	MF	P	MP	DMP	DBT	Total
Exposure															
0	-**	0.2	0.1	0.3	1.0	0.8	-	-	-	-	-	-	-	-	2.4
8	235	14.7	8.7	15.0	21.8	9.1	0.3	0.5	1.0	1.2	1.9	1.9	0.3	0.3	311.7
Depuration															
3	156	12.0	8.4	12.0	22.7	10.8	0.3	0.4	0.7	0.7	1.3	1.3	0.2	0.3	227.1
6	68	7.3	5.1	7.3	13.2	5.7	0.1	0.2	0.4	0.2	0.6	0.6	0.1	0.1	108.9
24	18	6.5	5.7	7.6	14.8	9.5	0.2	0.2	0.5	0.7	1.2	1.3	0.3	0.2	66.7
120	10	4.7	4.7	6.8	13.4	4.9	0.1	0.1	0.2	0.1	0.4	0.4	0.2	0.1	46.1
672	-	-	-	0.1	0.5	0.9	-	-	-	-	-	-	-	-	1.5
No. 2 fuel oil (% composition)	7.38	0.4	0.82	1.89	3.11	1.84	0.16		0.36		0.53			0.07	16.56

*n-P, C_{12}-C_{24} n-paraffins; N, naphthalene; 1-MN, 1-methylnaphthalene; 2-MN, 2-methylnaphthalene; DMN, dimethylnaphthalenes; TMN, trimethylnaphthalenes; B, biphenyl; MB, methylbiphenyls; F, fluorene; MF, methylfluorenes; P, phenanthrene; MP, methylphenanthrenes; DMP, dimethylphenanthrenes; DBT, dibenzothiophene.

**less than 0.1 ppm

concentrations for more highly substituted naphthalenes, biphenyls, and fluorenes as well as for dibenzothiophenes and phenanthrenes were attained 100 hr post-spill. Many of the PAHs which reached peak concentrations at 25 hr were released to low or undetectable levels after 100 hr. Higher molecular weight PAHs were released to undetectable levels in 242 to 509 hr.

TABLE 8. CONCENTRATIONS OF PAH IN THE TISSUES OF OYSTERS, _CRASSOSTREA VIRGINICA_, EXPOSED TO A SPILL OF 85 ℓ OF NO. 2 FUEL OIL IN A 24 x 24 m ENCLOSED SHORELINE AREA NEAR YORKTOWN, VA. CONCENTRATIONS IN THE OYSTER IN PPM (μg/g wet wt) AND IN SEAWATER IN PPB (μg/ℓ) (FROM BIERI AND STAMOUDIS, 1977)

Compound	Time after spill							
	+6 hr		+25 hr		+100 hr		+242 hr	+509 hr
	water	oysters	water	oysters	water	oysters	oysters	oysters
2-Methylnaphthalene	11	0.13	0.2	0.15	-*	-	-	-
1-Methylnaphthalene	9	0.11	0.2	0.12	-	-	-	-
Biphenyl & 2,6-dimethylnaphthalene	8	0.20	0.2	0.28	-	-	-	-
1,3-Dimethylnaphthalene	11	0.25	0.3	0.35	-	-	-	-
1,5- & 2,3-Dimethylnaphthalene	3	0.07	-	0.13	-	-	-	-
3- & 4-Methylbiphenyl & C_3-naphthalene	2	0.10	-	0.16	-	-	-	-
C_3-naphthalenes	7	0.32	-	0.48	-	0.25	0.03	-
2,3,5-Trimethylnaphthalene & C_4-naphthalene	2	0.13	-	0.23	-	0.07	-	-
C_2-Biphenyl, C_3-naphthalene & C_4-naphthalene	1	0.07	-	0.19	-	-	-	-
Fluorene, C_4-naphthalenes, methylacenaphthenes & C_2-biphenyl	2	0.09	-	0.18	-	-	-	-
C_4-Naphthalene & C_2-biphenyl	0.4	0.04	-	0.09	-	0.13	0.05	-
C_4-Naphthalenes & C_5-naphthalene	0.5	0.05	-	0.13	-	0.37	0.13	-
C_5-Naphthalene, C_2-biphenyl & methylfluorenes	2	0.16	-	0.40	-	0.47	-	-
C_5-Naphthalene, C_2-biphenyl & C_3-biphenyl	-	0.08	-	0.14	-	0.24	0.10	-
C_6-Naphthalene, C_2-biphenyl, C_3-biphenyl, methylfluorene, & C_4-biphenyl	-	0.05	-	0.11	-	0.20	0.10	-
Dibenzothiophene, C_4-biphenyl, C_2-fluorene, & phenanthrene	2	0.20	-	0.71	-	1.41	0.56	-

TABLE 8 (CONTINUED)

Compound	Time after spill +6 hr		+25 hr		+100 hr		+242 hr	+509 hr
	water	oysters	water	oysters	water	oysters	oysters	oysters
Methyldibenzothiophene	0.3	-	-	0.18	-	0.32	0.17	-
Methyldibenzothiophene, C_3-fluorene, & 2-methylphenanthrene	1	0.07	-	0.33	-	0.32	0.27	-
1-Methylphenanthrene, C_2-dibenzothiophene & C_3-fluorene	1	0.05	-	0.17	-	0.34	0.17	-
C_2-Dibenzothiophene	0.1	0.02	-	0.06	-	0.18	0.13	-
3,6-Dimethylphenanthrene & C_2-phenanthrene	0.2	0.03	-	0.09	-	0.35	0.20	-
C_2-phenanthrenes & C_3-dibenzothiophene	0.4	0.10	-	0.27	-	0.77	0.48	-
C_3-Phenanthrene	-	0.03	-	0.04	-	0.21	0.15	-
Total unresolved envelope[1]		5		9		24	12	4

*, Compound either absent, below background or cannot be identified.

1, Semiquantitative estimate from unresolved area.

All PAHs reached maximum concentrations in the water in 6 hr, then decreased to low or undetectable levels after 25 hr. Bieri and Stamoudis suggested that continued increase of PAH concentrations in the tissues of oysters long after water-accomodated PAHs had disappeared indicated that oysters acquired PAHs from oil-contaminated organic detritus via the digestive tract. The authors went on to hypothesize that apparent PAH bioaccumulation factors and changes in PAH concentrations in the tissues of animals exposed to an oil spill in their natural environment are determined mainly by residence time of different PAHs in the environment. Hard shell clams accumulated only about one-tenth as much PAH as the oysters, indicating a substantial interspecific difference in ability of molluscs to accumulate petroleum hydrocarbons.

Bieri *et al*. (1977) used a similar experimental design to investigate accumulation of petroleum hydrocarbons from spills of new and weathered south Louisiana crude oil by the mummichog, *Fundulus heteroclitus*. The concentration of all n-alkanes in the fish reached a maximum 76 hr after a spill of fresh crude. Naphthalene, monomethylnaphthalenes, and 2,3-dimethylnaphthalene reached maximum concentrations in fish tissues 31 hr after the spill, while all other PAHs analyzed reached maximum concentrations after 76 hr (Table 9). All PAHs except naphthalene, 2-methylnaphthalene, and the dibenzothiophene-C_4-biphenyl-C_5-naphthalene group were detectable in fish tissues 216 hr post-spill. Nearly all PAHs reached a maximum concentration in the exposure water 31 hr after the spill. Naphthalene, methylnaphthalenes, and some dimethylnaphthalenes disappeared rapidly from the water between 31- and 76-hr samplings. Mean apparent bioaccumulation factors (concentration in tissues/concentration in water) were about 1000 at 31 hr for PAHs and 290 for aliphatics. At 76 hr, bioaccumulation factors were 2700 for PAHs and 220 for aliphatics. Bioaccumulation factors for alkyl naphthalenes were similar although higher than that for naphthalene, but there was no relation between degree of alkylation and bioaccumulation factor.

PAHs were accumulated by the fish much more rapidly from the weathered crude oil and all experimental fish died within 120 hr. The difference was partially explained by the observation that PAH were accomodated into the water column much more rapidly from weathered than fresh crude oil.

Although maximum concentrations of individual PAHs in the water following spills of crude oil (fish experiment) were much lower than maximum PAH concentrations in the water following the spill of No. 2 fuel oil (oyster experiment), the fish accumulated substantially higher concentrations of PAH than oysters. Mummichogs also tended to retain PAH in their tissues longer than oysters. Thus, ability to metabolize PAH (high in fish, low in oysters) may have little effect on the rate of PAH uptake and release in an acute oil-spill situation. The greater accumulation of PAH by mummichogs is probably attributable primarily to the fact that they could not escape from the contaminants, while oysters could remain isolated through valve closure from the contaminated medium for long periods.

In the experiments described above, animals were exposed to both dispersed oil droplets and water-soluble fractions of oil. It is likely that some PAHs measured in the animals were actually present as unassimilated micro-oil droplets in the gut or adsorbed to the gills or other body surfaces. This may partly account for the lack of differential uptake of various PAHs from the oil as opposed to exposure to water-soluble fractions (WSF) of oil alone where this form of uptake is sometimes observed. Rossi and Anderson (1977) reported that mature male polychaete worms, *Neanthes arenaceodentata*, accumulated higher concentrations of methylnaphthalenes than naphthalene and dimethylnaphthalenes during 8-hr exposure to a WSF of No. 2 fuel oil, although the WSF contained a higher concentration of naphthalene than of methylnaphthalenes. Gravid female worms accumulated more dimethylnaphthalenes than methylnaphthalenes and naphthalene. All naphthalenes were released at the same rate when the worms were returned to clean seawater. Neff et al. (1976b) reported 24-hr bioaccumulation factors of 2.3 naphthalene, 8.1 to 8.5 methylnaphthalenes, and 17.1 and 26.7 dimethyl- and trilmethylnaphthalenes for clams exposed to a WSF of No. 2 fuel oil. Cox and colleagues spilled No. 2 fuel oil on the surface of a shrimp mariculture pond and measured concentrations of naphthalenes in the water and tissues of several species of marine animals from the pond. Naphthalenes were greatly enriched in the tissues of crustaceans in comparison to aqueous concentrations (Table 10).

TABLE 9. CONCENTRATIONS OF PAH IN THE TISSUES OF MUIMMICHOGS, *FUNDULUS HETEROCLITUS*, EXPOSED TO A SPILL OF 570 ℓ OF SOUTH LOUISIANA CRUDE OIL IN A 810 m^2 ENCLOSED SHORELINE AREA NEAR YORKTOWN, VA. CONCENTRATIONS ARE IN PPM (μg/g wet wt.)(FROM BIERI *et al*., 1977)

Compound(s)	Time after spill			
	+6 hr	+31 hr	+76 hr	+216 hr
Naphthalene	0.25	0.68	0.14	-*
2-Methylnaphthalene	0.34	1.56	1.12	-
1-Methylnaphthalene	0.34	1.26	0.98	0.13
Biphenyl & 2,6-dimethylnaphthalene	0.25	0.81	0.99	0.25
1,3-Dimethylnaphthalene	0.36	1.18	1.54	0.40
1,5-Dimethylnaphthalene	0.13	0.37	0.47	0.16
2,3-Dimethylnaphthalene	0.04	0.05	0.03	0.05
3- & 4-Methylbiphenyl & C_3 naphthalene	0.10	0.27	0.56	0.14
C_3-Naphthalene	0.09	0.20	0.42	0.12
Methylbiphenyl & C_3-Naphthalene	0.07	0.17	0.44	0.09
2,3,5-Trimethylnaphthalene	0.07	0.13	0.25	0.08
C_3-Naphthalene & C_2-biphenyl	0.03	0.09	0.22	0.03
Fluorene, C_4- & C_5-naphthalenes & C_2-biphenyl	0.04	0.11	0.45	0.05
C_4-Naphthalene, C_2- & C_3-biphenyls	0.03	0.04	0.11	0.03
Methylfluorene, C_4-naphthalene & C_3-biphenyl	0.11	0.23	0.54	0.13
Dibenzothiophene, C_4-biphenyl & C_5-naphthalene	0.01	0.02	-	-
Phenanthrene	0.08	0.11	0.26	0.08

*Peak not detectable on the gas chromatogram.

Alkyl naphthalenes were more enriched than naphthalenes. There were quantitative differences in the amounts of naphthalenes accumulated by the three species, but the qualitative pattern of naphthalene and alkyl naphthalene uptake was similar. Coho salmon, Oncorhynchus kisutch, exposed to a dilute WSF of Prudoe Bay crude oil for five weeks, accumulated the more highly alkylated benzenes and naphthalenes faster than the less substituted aromatics (Table 11)(Roubal et al., 1977a). C_4- and C_5-benzenes and 2-methylnaphthalene had the highest bioaccumulation factors. Therefore, there is differential bioaccumulation of PAHs from water-soluble fractions of oil but not from dispersed oil.

TABLE 10. CONCENTRATIONS OF NAPHTHALENES IN THE WATER AND SELECTED CRUSTACEANS FROM AN EXPERIMENTAL SHRIMP POND TO WHICH NO. 2 FUEL OIL WAS ADDED. THE WATER SAMPLES WERE COLLECTED 24 HR AFTER THE EXPERIMENTAL SPILL AND THE TISSUE SAMPLES WERE COLLECTED 72 HR AFTER THE SPILL (FROM COX et al., 1975)

Sample	Mean Concentration (ppb)		
	Naphthalene	Methylnaphthalenes	Dimethylnaphthalenes
Water	2.3	15.4	15.2
Brown shrimp (Penaeus aztecus)	450	3,610	14,700
Fiddler crab (Uca minax)	750	4,520	16,800
Warf crab (Sesarma cinereum)	930	6,050	24,700

TABLE 11. CONCENTRATIONS OF AROMATIC HYDROCARBONS IN THE WSF OF PRUDOE BAY CRUDE OIL IN THE MUSCLE TISSUE OF YOUNG COHO SALMON, ONCORHYNCHUS KISUTCH. THE RESULTING BIOACCUMULATION FACTORS ARE ALSO INCLUDED. THE FISH WERE EXPOSED TO THE WSF AT 10° C FOR FIVE WEEKS IN A FLOW-THROUGH SYSTEM (FROM ROUBAL et al., 1977A)

Hydrocarbon	Concentration (ppb)		Bioaccumulation factor
	Water	Muscle Tissue	
C_2-Benzenes	352	490	1.39
C_3-Benzenes	40	1,500	37.5
C_4- & C_5-Benzenes	12	5,500	458.3
Naphthalene	4	240	60
1-Methylnaphthalene	4	400	100
2-Methylnaphthalene	4	560	140
C_2-Naphthalenes	10	850	85.0
C_3-Naphthalenes	6	680	113.3

Distribution of PAH in Tissues of Aquatic Animals

Patterns of accumulation and release of PAH by different body regions of oil-exposed aquatic animals also vary. When shrimp, *Penaeus aztecus*, were exposed to a dilute WSF of No. 2 fuel oil for 20 hr, maximum concentrations of naphthalenes were reached in the head region, abdomen, gill, and exoskeleton within the first hour of exposure (Figure 2)(Neff *et al*., 1976b). The digestive gland continued to accumulate naphthalenes for the full 20-hr exposure period and contained more than ten times the concentration of naphthalenes than other tissues analyzed at the end of the exposure period. When shrimp were returned to clean seawater, the abdominal muscle and exoskeleton released naphthalenes very rapidly to undetectable levels after a 25-hr depuration. The head region released naphthalenes more slowly. Nearly 250 hr were required for complete depuration of naphthalenes from the gill and hepatopancreas, suggesting that in shrimp the hepatopancreas is an important site of PAH storage and metabolism and the gills are an important route of PAH excretion.

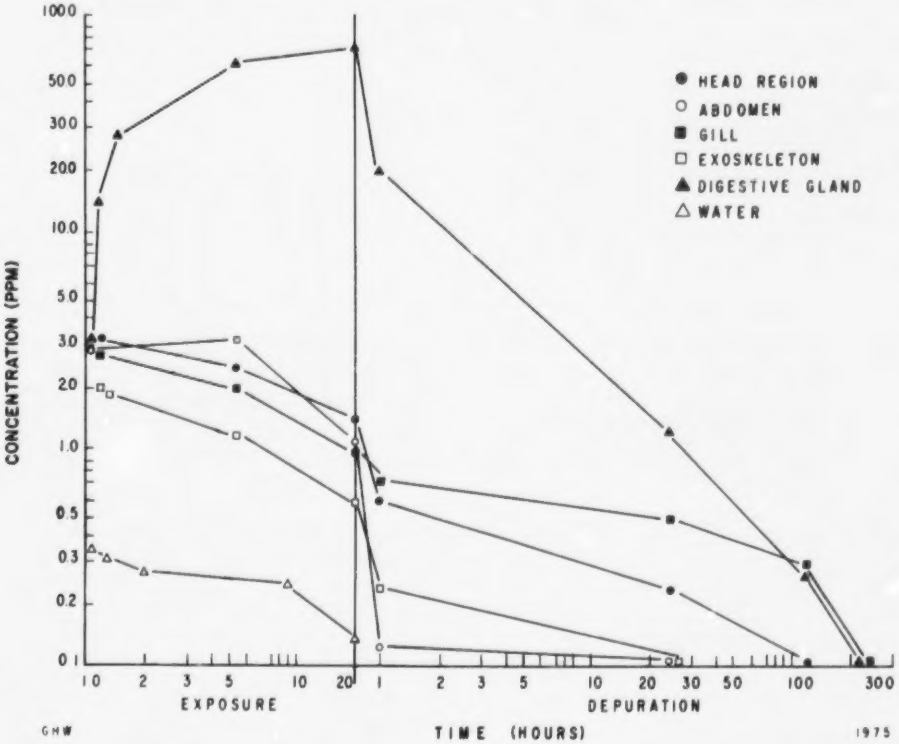

Figure 2. Accumulation and retention of naphthalenes (naphthalene, methylnaphthalenes, and dimethylnaphthalenes) by different body regions of juvenile brown shrimp, *Penaeus aztecus*, exposed to a 20% dilution of the water soluble fraction of No. 2 fuel oil (From Neff *et al*., 1976b).

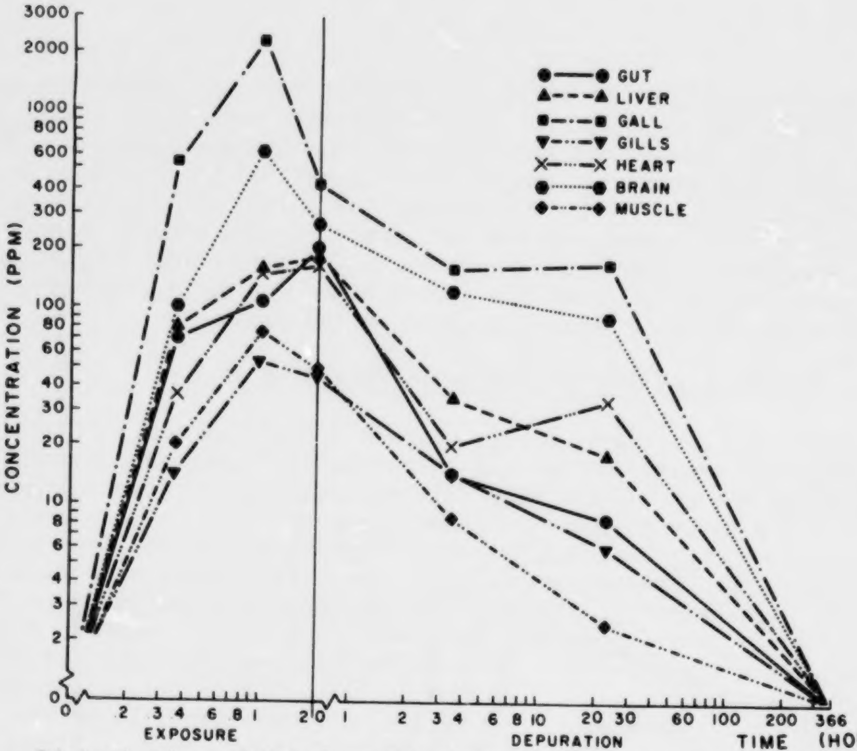

Figure 3. Distribution of total naphthalenes in the tissues of Gulf killfish, Fundulus similis, during exposure to the water soluble-fraction of No. 2 fuel oil and at different times following exposure (From Neff et al., 1976b).

Naphthalenes also accumulated very rapidly in Gulf killifish, Fundulus similis, during 2-hr exposure to the WSF of No. 2 fuel oil (Figure 3)(Neff et al., 1976b). Maximum naphthalene concentrations were reached in most tissues after 1-hr exposure. The gallbladder and brain contained the highest concentrations of total naphthalenes (2300 and 620 ppm, respectively, after 1 hr). When fish were returned to clean seawater, all organ systems immediatly began to release naphthalenes. The somatic muscles released naphthalenes most rapidly, whereas the gallbladder and brain released naphthalenes much more slowly. Complete depuration of naphthalenes from all tissues required 366 hr. Slightly different results were obtained when pink salmon fry, Oncorhynchus gorbuscha, were exposed to a WSF of Cook Inlet crude oil for four days (Rice et al., 1977). Maximum concentrations of naphthalenes were reached in gut, gill, and skeletal muscle (the only tissues analyzed) after 10-hr exposure; the highest concentration of naphthalene was found in the gut at all sampling times, the next highest in skeletal muscle tissue. Methylnaphthalenes were released more rapidly than dimethylnaphthalenes from the gut during the latter part of the exposure period and after fish were returned to clean seawater.

Mallard ducks, Anas platyrhynchos, fed 5 mℓ of south Louisiana crude oil per day for 14 days demonstrated high concentrations of aromatic hydrocarabons in their tissues (Lawler et al., 1978). The skin and underlying adipose tissue accumulated greater concentrations of aromatic hydrocarbons than did other tissues examined including liver, breast muscle, heart muscle, brain, uropygial gland, and blood. Two- and three-ring aromatics were accumulated to a greater extent than were benzenes. Ringed seals, Phoca hispida, rapidly accumulated petroleum hydrocarbons, including aromatics, when exposed to Normal Wells crude oil by immersion or ingestion (Engelhardt et al., 1977). Relatively low levels of hydrocarbons were detected in such tissues as blubber, brain, liver, kidney, muscle, and lung. Somewhat higher levels were found in whole blood. Hydrocarbon concentrations in the bile and urine were high, indicating that these were major routes of hydrocarbon excretion by the seals.

Effect of Dissolved Organic Matter on Uptake of PAHs

Marine waters nearly always contain significant amounts of dissolved and colloidal organic matter and suspended inorganic and organic particulate matter. These materials may interact with petroleum or PAH entering the water column in such a way as to change bioavailability of these compounds to marine organisms. Sanborn and Malins (1977) reported that ^{14}C-naphthalene was accumulated nearly four times more rapidly than ^{14}C-naphthalene complexed to bovine serum albumen by stage V spot shrimp, Pandalus platyceros. Dunn and Young (1976) inferred from their data on BaP contamination in mussels, Mytilus edulis, from California that BaP associated with particulate matter of pyrolytic origin was not readily available to marine molluscs. Studies on bioavailability of PAH from food and sediments also seem to indicate that, in most cases, PAH complexed to colloidal organic materials or adsorbed to organic or inorganic particulates are less bioavailable than PAH in solution or fine dispersion in water. Several types of dissolved organic compounds may actually increase the solubility or accomodation of PAHs and other petroleum hydrocarbons in water. Humic substances are complex organic macromolecules of plant origin commonly found in fresh and coastal marine waters in soluble or colloidal form. Because of their detergency, they tend to increase accomodation of poorly soluble organic materials in water. Boehm and Quinn (1976) studied the effect of natural dissolved organic matter (DOM), primarily humic substances, from Narragansett Bay, RI, on the bioavailability of selected petroleum hydrocarbons and No. 2 fuel oil to the hard shell clam, Mercenaria mercenaria. Removal of DOM from seawater significantly increased uptake of n-hexadecane from water. No significant difference was evident in uptake of phenanthrene from seawater containing DOM compared to seawater from which DOM had been removed. The clams accumulated, on the average, seven times more total hydrocarbons from dispersions of No. 2 fuel oil in DOM-free seawater than from dispersions in seawater containing natural levels of DOM. In the absence of DOM, uptake of saturated hydrocarbons from fuel oil was increased 17-fold whereas uptake of aromatic hydrocarbons was increased 5-fold. Because of extremely low aqueous solubilities, alkanes above about C_6 are present in water primarily as finely dispersed droplets (McAuliffe, 1966). Addition of DOM either increases their solubility or decreases droplet size so that alkanes can pass through the gill filter of Mercenaria. Dispersed oil droplets would behave similarly to alkanes. However, phenanthrene is sufficiently soluble at the concentrations used (1 mg/ℓ) and therefore its physical state in the water would not be affected by presence of DOM.

Accumulation of PAH from Sediment

Bottom sediments of lakes, rivers, and coastal marine waters are the ultimate repository for much of the PAH entering the aquatic environment from various sources. Large populations of microbes, plants, and animals live on the surface of or within bottom sediments, particularly in estuarine and coastal marine environments. Some benthic animals actually ingest bottom sediments and remove organic materials from them as a source of nutrition (deposit feeder). The question of bioavailability of sediment-adsorbed PAH to these benthic organisms has received relatively little attention. This is unfortunate since assimilation of PAH from sediments by benthic organisms would provide a mechanism by which PAH could be cycled from sediments into the aquatic food chain. In addition, bioaccumulation of PAH from sediments by benthic organisms might pose a health hazard to the predators (including man) of benthic organisms.

Rossi (1977) exposed the marine polychaete worm, *Neanthes arenaceodentata*, to sediment contaminated with No. 2 fuel oil. Total concentrations of naphthalenes in the sediment dropped from 9 to 3 ppm during the 38-day exposure. However, naphthalenes concentrations in water of the flow-through exposure system never rose above the 0.01 ppm detection limit. Analysis of more than 20 replicate samples of worm tissues, taken periodically throughout the exposure period, showed that the polychaetes contained less than 0.1 ppm total naphthalenes at all sampling times. The worms were apparently unable to accumulate naphthalenes from the sediment although they were observed to ingest sediment and pass it through their digestive tracts.

Anderson *et al*. (1977) obtained similar results when they exposed the sipunculid worm, *Phascolosoma agassizii*, to sediments contaminated with Prudoe Bay crude oil. Total hydrocarbon concentration in the sediment ranged from 475 to 765 ppm and total naphthalenes from 2.6 to 3.7 ppm during the two-week exposure period. The highest mean concentrations of total naphthalenes in the worms were 3.8 to 4.8 ppm, reached after 40-hr exposure. Worms exposed to contaminated sediment for two weeks contained a mean 1.9 ppm total naphthalenes. When worms were returned to clean sediment, they rapidly released accumulated naphthalenes to undetectable levels in two weeks or less. Sipunculids never contained naphthalene concentrations significantly higher than those in the contaminated sediment, and much of the hydrocarbon present in the worms was probably associated with sediment in the gut. The authors concluded that the sipunuclid worms were unable to accumulate naphthalenes from sediment to a significant extent.

Fucik *et al*. (1977) transferred the clam, *Rangia cuneata*, to sediments at several stations in the vicinity of an oil separator platform in Trinity Bay, TX. The bottom water near the platform contained 0.19 ppb total naphthalenes and bottom sediments contained 27.7 ppm total naphthalenes. Clams accumulated up to 33.6 ppm total naphthalenes in 97 days. There was a good correlation between rates of uptake of naphthalenes by the clams and levels of naphthalenes in sediments at the different stations. Gas of clam extracts showed a large unresolved envelope of weathered petroleum similar to that in sediments. The authors concluded that at least part of the naphthalenes accumulated by *Rangia* was derived from sediments. The majority of tissue samples contained lower concentrations of naphthalenes than did sediments, indicating that uptake of naphthalenes from sediments by *Rangia* was very inefficient.

Accumulation of naphthalenes from oil-contaminated sediment and detritus by the detritivorous clam, *Macoma inquinata*, was investigated by Roesijadi *et al*. (1978b). Sandy sediments artificially contaminated with Prudoe Bay crude oil contained initial concentrations of 90.2 to 2750.8 ppm total hydrocarbons and 0.45 to 11.96 ppm total naphthalenes. Total hydrocarbon and naphthalene concentrations in the sediments decreased by 44.6 and 88.8%, respectively, during the 15-day exposure period. Tissues of sediment-exposed clams contained 0.01 to 0.15 ppm total naphthalenes compared to 0.01 to 0.07 ppm in controls. Thus, the clams were unable to accumulate naphthalenes from the heavily contaminated sediment. Clams exposed to detritus contaminated with Prudoe Bay crude oil spiked with ^{14}C-2-methylnaphthalene failed to accumulate significant concentrations of ^{14}C-2-methylnaphthalene from the detritus. All of the 2-methylnaphthalene in clam tissues could be accounted for via uptake from seawater, presumably of solubilized material or of ^{14}C-methylnaphthalene adsorbed to very fine suspended matter.

Roesijadi *et al*. (1978a) studied accumulation of Prudoe Bay crude oil and specific PAHs from oil-contaminated sediments by three benthic infaunal invertebrate species: the sipunculid worm, *Phascolosoma agassizii*, and clams, *Macoma inquinata* and *Protothaca staminea*. *P. agassizii* and *M. inquinata* are deposit feeders while *P. staminea* is a suspension feeder. The animals were exposed on the lower shore of Sequim Bay, WA, in boxes containing beachsand contaminated with Prudoe Bay crude oil. Total hydrocarbon concentrations in the exposure sediment were 887.4 μg/g initially and declined to 443.8 and 420.6 μg/g after 40 and 60 days in the field. Hydrocarbon accumulation was generally greater in the two deposit feeding species than in the suspension feeder (Table 12). The amounts of aliphatic and diaromatic hydrocarbons accumulated by all species were quite low even after 60 days of exposure to contaminated sediment. *M. inquinata* accumulated up to 2.24 to 2.68 ppm total naphthalenes (mostly dimethylnaphthalenes) in 60 days. These results again illustrate the very limited bioavailability of sediment-adsorbed petroleum hydrocarbons even to sediment-ingesting benthic invertebrates.

Clams, *M. inquinata*, were also exposed in the laboratory to sediments containing detritus contaminated with Prudoe Bay crude oil and spiked with four different ^{14}C-PAH. One group of clams was exposed directly to the sediment, while another group was suspended in the overlying water column. This allowed the investigators to estimate the relative efficiency and magnitude of PAH uptake from detritus and water. After seven days, the sediment, water, and clams were analyzed for ^{14}C activity. Efficiency of PAH uptake from sediments was much lower than from water (Table 13). Bioaccumulation factors for uptake of the four PAHs from contaminated sediments were 0.2 or less indicating no significant bioaccumulation of PAH by this route. Bioaccumulation factors for uptake of the four PAHs from seawater were in the 10.3 to 1349 range indicating a significant potential for bioaccumulation, particularly of higher molecular weight PAHs, from seawater. Thus, accumulation of PAH from sediment, when it occurs at all, may be attributed in large part to uptake of PAH desorbed from sediment particles into the interstitial water.

TABLE 12. CONCENTRATIONS OF ALIPHATIC AND DIAROMATIC HYDROCARBONS IN THE TISSUES OF THREE BENTHIC INFAUNAL INVERTEBRATES, *PHASCOLOSOMA AGASSIZII*, *MACOMA INQUINATA*, AND *PROTOTHACA STAMINEA*, FOLLOWING EXPOSURE IN THE FIELD FOR 40 OR 60 DAYS TO SEDIMENT CONTAMINATED WITH PRUDOE BAY CRUDE OIL AT AN INITIAL CONCENTRATION OF 887.4 μg TOTAL HYDROCARBONS/g SEDIMENT (FROM ROESIJADI *et al*., 1978A)

Species	Treatment	Hydrocarbon (μg/g wet wt)*				
		C_{12}-C_{28}	N	MN	DMN	TN
P. agassizii	Control	<0.10	<0.005	<0.005	<0.01	<0.02
M. inquinata	Control	<0.10	<0.005	<0.005	<0.01	<0.02
P. staminea	Control	<0.10	<0.005	<0.005	<0.01	<0.02
P. agassizii	40 day exp.	1.90	<0.005	0.23	0.60	0.83
P. agassizii	40 day exp.	0.73	<0.005	0.01	0.15	0.16
M. inquinata	40 day exp.	0.69	<0.005	0.06	0.89	0.95
P. staminea	40 day exp.	<0.10	<0.005	<0.005	<0.01	<0.02
P. agassizii	60 day exp.	1.48	0.01	0.06	0.18	0.25
M. inquinata	60 day exp.	0.54	0.02	0.27	2.39	2.68
M. inquinata	60 day exp.	3.62	0.02	0.26	1.96	2.24
P. staminea	60 day exp.	0.10	<0.005	0.02	0.16	0.18

*C_{12}-C_{28}, C_{12}-C_{28}n-paraffins; N, naphthalene; MN, methylnaphthalenes; DMN, dimethylnaphthalenes; TN, the sum of naphthalene, methylnaphthalenes and dimethylnaphthalenes.

Recently, McCain *et al*. (1978) reported the accumulation of aromatic hydrocarbons from experimentally oiled sediments by the English sole, *Parophrys vetulus*. The flatfish was exposed for four months to natural marine sediments experimentally contaminated with Alaskan North Slope crude oil to an initial level of 700 μg/g dry weight. Aromatic hydrocarbons accumulated rapidly in the tissues of the fish and reached maximum levels in the three tissues examined after 11 days (Table 14). The liver contained higher concentrations of all aromatics detected than did skin or muscle. After 27 days, only the liver contained significant concentrations

TABLE 13. ACCUMULATION OF ^{14}C-PAH FROM SEDIMENT BY THE DEPOSIT-FEEDING CLAM, MACOMA INQUINATA. CLAMS WERE EXPOSED TO SEVEN DAYS TO SEDIMENT CONTAINING AN INITIAL CONCENTRATION OF 2000 μg/g PRUDOE BAY CRUDE OIL SPIKED WITH 10 μ CI OF THE PAH INDICATED (FROM ROESIJADI *et al.*, 1978A)

Parameter	PAH*			
	Phe	Chry	DMBA	BaP
Net uptake from sediment (μg/g)	0.096	0.308	0.297	0.059
Uptake from seawater (μg/g)	0.038	0.297	0.856	0.037
Concentration in sediment at seven days (μg/g)	0.49	8.37	4.63	0.64
Concentration in seawater at seven days (μg/ml)	3.7×10^{-3}	4.3×10^{-4}	6.3×10^{-4}	4.3×10^{-5}
Bioaccumulation factor for uptake from sediment**	0.20	0.04	0.06	0.09
Bioaccumulation factor for uptake from seawater***	10.3	694	1349	861

*Phe, phenanthrene; Chry, chrysene; DMBA, dimethylbenz[a]anthracene; BaP, benzo[a]pyrene.

**Calculated as net uptake from sediment/concentration in sediment at seven days.

***Calculated as net uptake from seawater/concentration in seawater at seven days.

TABLE 14. AROMATIC HYDROCARBONS IN THE TISSUES OF ENGLISH SOLE, PAROPHRYS VELULUS, EXPOSED CONTINUOUSLY FOR TWO MONTHS TO SEDIMENT CONTAMINATED WITH ALASKAN NORTH SLOPE CRUDE OIL (FROM MCCAIN *et al.*,1978)

Compound	Concentration (ng/g dry wt)								
	11 days			27 days			51 days		
	skin	muscle	liver	skin	muscle	liver	skin	muscle	liver
1,2,3,4-tetramethylbenzene	33	44	922	-	-	863	-	-	124
Biphenyl	156	-	307	-	-	278	-	-	-
Naphthalene	82	20	100	-	-	-	-	-	-
1-Methylnaphthalene	1189	369	1940	-	-	1325	-	-	-
2-Methylnaphthalene	888	279	3070	-	-	1500	-	-	60
2,6-Dimethylnaphthalene	130	-	69	-	-	-	-	-	-

of aromatic hydrocarbons and after 51 days the liver contained only 124 ppb 1,2,3,4-tetramethylbenzene and 60 ppb 2-methylnaphthalene. The concentrations of aromatics found in the tissues of the fish throughout the experiment were of the same order of magnitude as aromatic hydrocarbon concentrations in the oiled sediment. However, trimethylnaphthalene, fluorene, and phenanthrene, although present in the oiled sediment, were not detected in the tissues of the fish. Thus, accumulation of aromatic hydrocarbons from oiled sediment by fish is substantially less efficient than uptake from the water.

All studies discussed above lead to the general conclusion that sediment-adsorbed PAHs are not readily assimilated by benthic animals. However, relatively few species have beeen studied. Nothing is known about PAH uptake from sediments by deposit-feeding fish (e.g., mullet) or attached aquatic plants.

Effects of Endogenous and Exogenous Factors on the Accumulation and Release of PAH by Aquatic Organisms

Several endogenous biological factors such as size, nutritional status body composition, age, and sex, as well as exogenous physical factors such as salinity and temperature, may affect patterns of uptake and release of PAH by aquatic organisms. Relatively few investigations have been performed in these areas. Studies of this sort might provide valuable information on mechanisms of PAH accumulation and retention by aquatic organisms.

Endogenous Factors

Stegeman and Teal (1973) observed that oysters, Crassostrea virginica, containing high concentrations of tissue lipids accumulated petroleum hydrocarbons to higher concentrations than did low fat oysters. Several other investigators have noted a positive correlation between lipid content of marine organisms and their ability to accumulate petroleum hydrocarbons, including PAH, from water. Harris et al. (1977a) studied accumulation of ^{14}C-naphthalene by nine species of marine and estuarine copepods. Retention of ^{14}C-naphthalene following 24-hr exposure to this PAH in solution varied nearly 16-fold among the different copepod species. Strong positive correlations were drawn between body weight, ash-free dry weight or total lipid content, and naphthalene retention, especially between total lipid content and naphthalene retention. The authors went on to study the uptake and retention of ^{14}C-naphthalene by male and female Calanus helgolandicus which had either been starved for five days or fed Biddulphia cells before the uptake experiment began. Starved male and female copepods showed significantly lower levels of both total lipid and lipid as percent of ash-free dry weight than did fed copepods. Starved animals also accumulated significantly less ^{14}C-naphthalene than fed individuals. Interestingly, both starved and fed male copepods contained nearly three times as much total lipid as starved and fed females, yet males and females accumulated roughly equivalent concentrations of ^{14}C-naphthalene. It was suggested that lipid composition of males and females may be different and that these compositional differences may affect naphthalene retention by each sex. Although ^{14}C-naphthalene uptake and retention from solution was positively correlated with total lipid content of the copepods, rate assimilation of naphthalene from food was unrelated to total lipid content of the animals. Lee (1975) observed that rate of accumulation of ^{3}H-BaP was significantly greater in large than small copepods.

Mature male and gravid female polychaete worms, *Neanthes arenaceodentata*, accumulated naphthalenes at a similar rate from the water-soluble fraction of No. 2 fuel oil (Rossi and Anderson, 1977). However, males rapidly released naphthalenes when returned to oil-free seawater, while gravid females retained them until spawning occurred about 300 to 500 hr after the beginning of the depuration period. Newly released zygotes contained high concentrations of naphthalenes and retained them during early developmental stages to the trochophore stage several days later. The trochophore and later juvenile stages rapidly released naphthalenes. Gravid female *Neanthes* contain high concentrations of lipids, primarily associated with the ovaries and developing eggs. Naphthalenes apparently accumulated in these gonadal lipid stores and were released in eggs at spawning. Yolk lipid stores are not utilized in this species until larvae reach the trochophore stage. When the lipids were mobilized, naphthalenes were released rapidly. Thus, hydrocarbons, which become associated with stable lipid pools, such as depot lipids and gonadal-lipid stores, may be retained until the animals mobilize lipids for nutritional purposes. Hydrocarbons, which become associated with more labile hydrophobic compartments, such as membrane lipids and cellular macromolecules, may be released rapidly when ambient levels of hydrocarbons decrease. Such a two-compartment model may partially explain the observation of several investigators that depuration of chronically accumulated hydrocarbons is a two-phase process characterized by an initial rapid release of hydrocarbons followed by a second phase of very gradual release of remaining hydrocarbons.

Exogenous Factors

Temperature and salinity of the ambient medium have a profound effect on may physiological functions in marine organisms. These factors also affect solubility, adsorption-desorption kinetics, octanol/water partition coefficients, etc., of PAH in water. Therefore, one would expect that salinity and temperature would have a significant effect on accumulation and release of PAH by marine organisms. Nevertheless, little research has been done in this area.

Young (1977) acclimated groups of estuarine grass shrimp, *Palaemonetes pugio*, to salinities of 5, 15, and 35 °/oo (parts per thousand) or 2, 17, and 32 °/oo S. Each group was then exposed to either 3 ppm naphthalene or 0.3 ppm phenanthrene at the acclimation salinity. Naphthalene uptake was rapid at all salinities. Tissue naphthalene concentrations reached a maximum in 2 hr. The highest level (57 ppm) was observed in animals acclimated and exposed at the intermediate salinity, 15 °/oo S. At 5 and 35 °/oo S, maximum naphthalene uptake was much less with 2-hr values of 27.5 and 41 ppm, respectively. Similar results were obtained in the phenanthrene exposure. Phenanthrene uptake was maximal (14.7 ppm) at the intermediate salinity and significantly lower at 2 and 32 °/oo S (both approximately 9.8 ppm)(Figure 4). When returned to phenanthrene-free seawater of the acclimation salinity, all three groups of shrimp released phenanthrene rapidly and at approximately the same rate. *P. pugio* is an excellent osmoregulator over its entire normal environmental salinity range. Body fluids are regulated hyperosmotic to the medium at low salinities and hypoosmotic to the medium at high salinities (Roesijadi *et al.*, 1976). At intermediate salinities in the 15 to 17 °/oo range, body fluids of the shrimp are isomotic to the medium. Water turnover rate measurements show that permeability of *P. pugio* is greatest at the isosmotic salinity and is reduced at salinities which are associated with active osmoregulation. These changes in apparent permeability of the shrimp are reflected in changes in rate of uptake but not release of PAHs at different salinities.

By comparison, salinity had only a marginally significant effect on rate of naphthalenes uptake from a WSF of southern Louisiana crude oil by the estuarine marsh clam, *Rangia* cuneta (Fucik and Neff, 1977). Naphthalenes uptake was lowest at 30 °/oo S and variable at lower slainities (Figure 5). *R. cuneata* is an osmoconformer throughout most of its normal salinity regime and osmoregulates only at salinities below about 5 °/oo (Bedford and Anderson, 1972). For another clam, *Protothaca staminea*, which is an osmoconformer throughout its entire environmental salinity regime, salinity did not have a statistically significant effect on uptake of naphthalenes from the WSF of southern Louisiana crude oil. Therefore, physiological processes underlying osmoregulation by aquatic animals may influence bioavailability to the animals of PAH in solution.

Temperature had a highly significant effect on rate of uptake of naphthalenes from a WSF of southern Louisiana crude oil by both *Rangia cuneta* and *Protothaca staminea* (Figure 5)(Fucik and Neff, 1977). Rate of naphthalenes uptake was highest at the lowest temperature used and decreased with increasing temperature. This inverse relationship between temperature and naphthalene uptake rate was not due to the influence of temperature on filtration rate of clams or on residence time of naphthalenes in the water. Filtration rate of *R. cuneata* (the rate at which water was pumped over the gills) increased in a nearly linear fashion with increasing temperature, so that the gills presumed to be the major sites of PAH uptake by bivalve molluscs (Lee *et al.*, 1972a) were actually exposed to larger volumes of the WSF at higher temperatures where naphthalenes uptake was lowest. Temperatures in the range used in these experiments did not have a statistically significant effect on initial concentration or residence time of naphthalenes in the exposure water. Rate of naphthalene release from oil-contaminated clams returned to clean seawater was not significantly affected by temperature. The influence of temperature on rate of accumulation of ^{14}C-naphthalene and ^{14}C-2-methylnaphthalene from solution by *R. cuneata* was also investigated with similar results (Fucik and Neff, unpublished observations): There was nearly a linear inverse relationship between ^{14}C uptake and temperature.

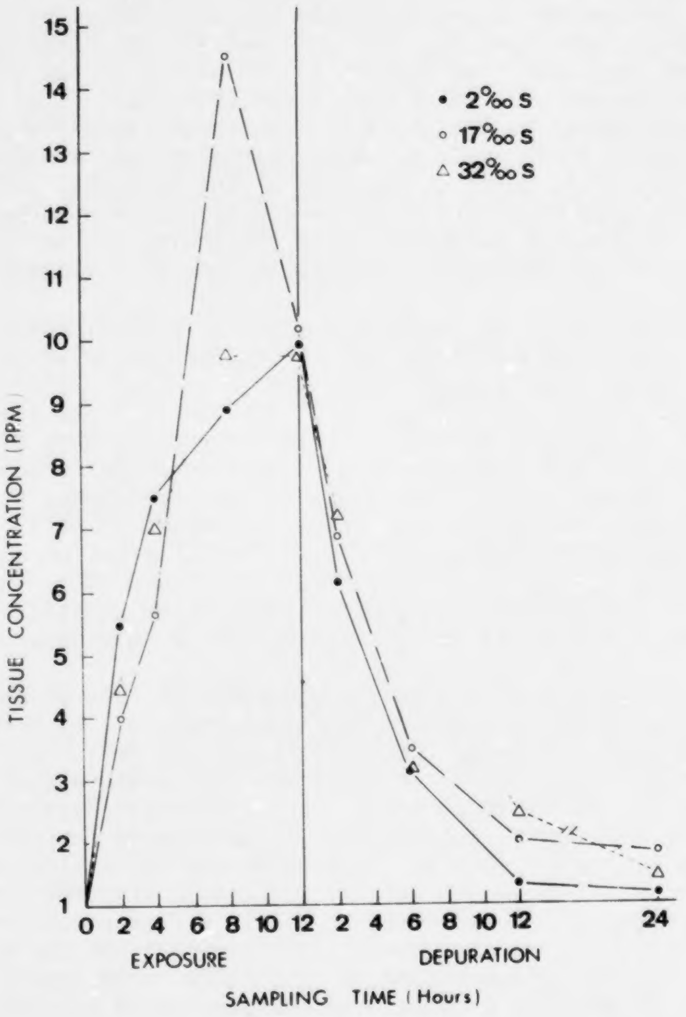

Figure 4. Accumulation and retention of phenanthrene by grass shrimp, Palaemonetes pugio, which were acclimated to and exposed to 0.3 ppm phenanthrene at salinities of 2, 17 or 32°/∘∘ (From Young, 1977).

Harris *et al*. (1977a) also demonstrated an inverse relationship between temperature and amount of ^{14}C-naphthalene accumulated by the copepod, *Calanus helgolandicus*, during exposure to 1 µg/ℓ ^{14}C-naphthalene for 24 hr (Figure 6). Accumulation of ^{14}C-naphthalene in each copepod decreased by about 39 pg (picograms), or by 3.23 pg/µg copepod lipid, per 10° C rise in temperature. The authors suggested that the effect of temperature on ^{14}C-naphthalene accumulation could be explained if rate of hydrocarbon metabolism increased more rapidly than rate of uptake as temperature increased. This would not explain the effect of temperature on naphthalenes accumulation by molluscs because they have little if any ability to metabolize naphthalenes. A better explanation of this phenomen is provided by Herbes (1977). He measured the effect of temperature on the adsorption of anthracene to non-living yeast cells from solution (0.02 µg/ℓ). The fraction of anthracene adsorbed by the yeast cells decreased significantly with increasing temperature. Calculated heat of adsorption for this process was 5.2 kcal/mole which is characteristic of simple physical (Van der Waals) adsorption. As temperature rises, the strength of this weak chemical bond decreases, favoring desorption of PAH from the particles Because partitioning of PAH between soluble and adsorbed phases is determiined by relative rates of adsorptive and desorptive reactions, adsorption will be increasingly favored over desorption as temperature decreases. Therefore, temperature exerts its effect on PAH uptake primarily at the initial step of the process--adsorption of PAH from water onto the surface of a biological membrane.

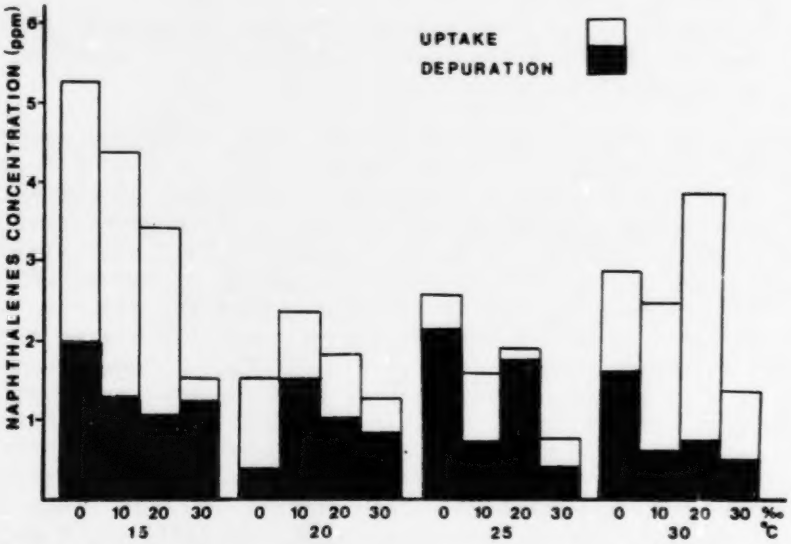

Figure 5. Uptake and release of total naphthalene by the clam, *Rangia cuneata*, exposed to a 25% water-soluble fraction of south Louisiana crude oil following acclimation for 14 days to different combinations of salinity and temperature (From Fucik and Neff, 1977).

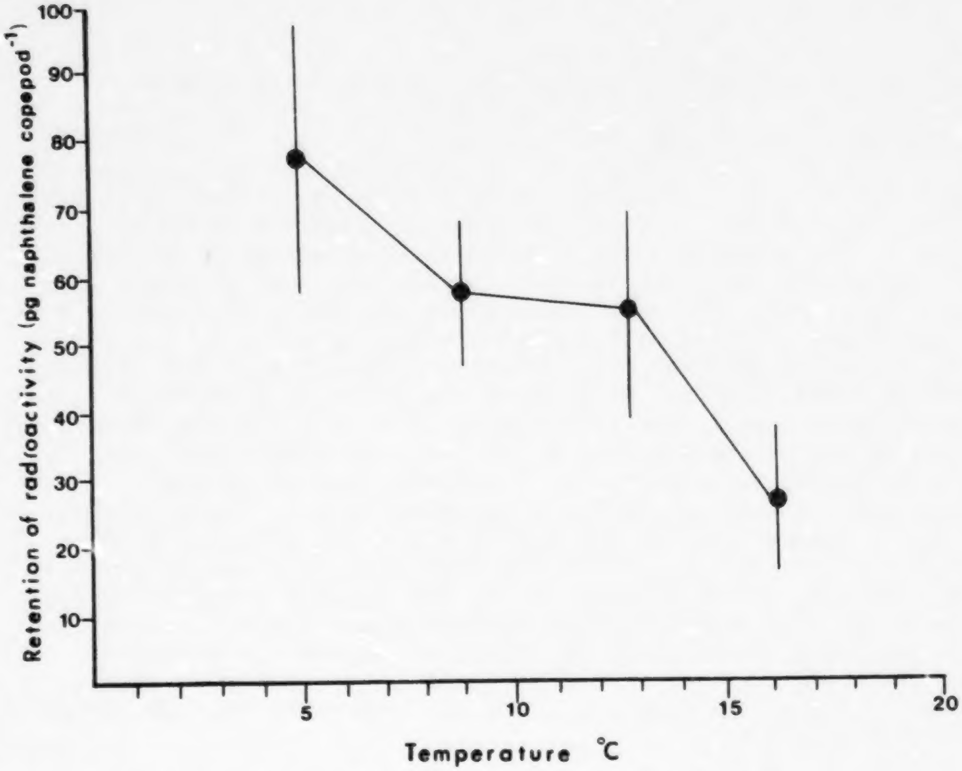

Figure 6. Effect of temperature on the accumulation of ^{14}C-naphthaleneby the copepod, Calanus helgolandicus, during exposure for 24 hr to 1 μg ^{14}C-naphthalene/ℓ seawater (From Harris et al., 1977a).

From the very limited research in this area, it is apparent that many biotic and abiotic factors influence rates and patterns of PAH uptake, retention, and release by marine organims. Since many of these variables are not controlled in laboratory hydrocarbon uptake studies, and none are controlled in field studies, it is not surprising that published rates of hydrocarbon uptake and release by marine organisms are so variable. For instance, several investigators have reported that the majority of hydrocarbons accumulated by marine animals are released rapidly when the animals are returned to clean seawater (Lee et al., 1972a,b; Stegeman and Teal, 1973; Neff et al., 1976b), whereas others have reported that hydrocarbons, once accumulated, are released very slowly, if at all, even after long periods of depuration (Blumer et al., 1970; Boehm and Quinn, 1977). These differences may be attributed to differences in such factors as the duration of exposure to hydrocarbons, lipid content and nutritional status of experimental animals, age and sex of animals, and salinity, temperature, and other physical environmental factors during the exposure and depuration periods.

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SOME ASPECTS OF THE UPTAKE AND ELIMINATION OF THE POLYNUCLEAR AROMATIC HYDROCARBON CHRYSENE BY MANGROVE SNAPPER, *LUTJANUS GRISEUS*, AND PINK SHRIMP, *PENAEUS DUORARUM*

by

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ABSTRACT

The accumulation of the polynuclear aromatic hydrocarbon (PAH), chrysene (found in shale and crude oil), was studied in the mangrove snapper, *Lutjanus griseus*, and its accumulation and elimination were studied in pink shrimp, *Penaeus duorarum*. When exposed to 1 and 5 μg/ℓ chrysene in a closed seawater environment, snapper were found to concentrate the contaminant in their livers, but not in other tissue (gallbladder, white muscle, intestine). The shrimp accumulated chrysene in both the cephalothorax and the abdomen. After exposure to chrysene for 28 days, shrimp transferred to fresh seawater released most of the contaminant rapidly, but detectable amounts remained in their bodies 28 days after the transfer.

INTRODUCTION

Spiraling energy needs of the United States have put a strain on presently exploited petroleum resources, requiring the petroleum industry to continue a search for oil farther offshore in deeper waters. Expanded oil production from offshore wells and the installation of the offshore deep-water ports off the coast of Texas and Louisiana have increased the potential for oil spills in the marine environment. According to NAS (1975), oil released in offshore production accidents represent about 1.3% (72.6 million kg/year) of the total 5.5 billion kg/year discharged in oceans. Oil spillage could increase to 181.4 million kg/year by the early 1980s.

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Moreover, offshore oil platforms attract large communities of marine species comprising the lower as well as higher trophic levels. The most productive shrimping grounds in the northern Gulf of Mexico are also major oil producing areas. Consequently, shrimp and fish attracted to rig structures are extremely vulnerable to both acute and chronic exposure to petroleum hydrocarbons. Since petroleum contains carcinogenic aromatic hydrocarbons (Mamedov, 1959; Gilchrist _et al_., 1972; McKay and Latham, 1973; Hurtubise, 1977), contamination of commercial fish species--such as shrimp and snapper--may pose a potential health hazard to consumers of marine foods. Other researchers have proven that upon exposure to certain polynuclear aromatic hydrocarbons (PAHs), bivalves (Cahnmann and Kuratsune, 1957), other invertebrates (Koe and Zechmeister, 1952; Corner _et al_., 1973; Rossi and Anderson, 1977), as well as some fish (Neff _et al_., 1976), accumulate these contaminants in certain tissues.

The purposes of our study were two-fold. The first was to determine if the carcinogen chrysene (Hecht _et al_., 1974) found in shale (Lahe and Eisen, 1968) and crude oils (Mamedov, 1959) is accumulated by two commercially important marine organisms--pink shrimp, _Penaeus duorarum_, and mangrove snapper, _Lutjanus griseus_--after exposure to 1 and 5 μg/ℓ concentrations of the contaminant in a closed seawater environment. The second was to determine which tissues, if any, accumulate chrysene and how rapidly the contaminant is eliminated after the organisms are transferred to fresh seawater. Chrysene was chosen for its stability and relatively low danger to the researchers.

MATERIALS and METHODS

Extraction

All solvents used were UV grade (Burdick and Jackson, Inc.*). The extraction procedure for both water and tissue samples was a modification of the technique developed by Bligh and Dyer (1959). In extracting chrysene from seawater samples, a system of methanol, chloroform, and water (sample) was established in the proportion 2:1:0.8 (v/v/wt) in a separatory funnel. Then the solution was shaken vigorously for 2 min, 1 volume of chlorofrom was added, the solution was shaken for 30 sec, 1 volume of distilled water was added, and the solution was shaken again for 30 sec. The phases were allowed to separate; the chloroform phase was removed, filtered through glass wool, and concentrated via rotoevaporation for analysis. This technique resulted in 90 to 95% recovery of chrysene.

For recovery of chrysene from tissue samples, whole lipid extracts were prepared. The wet tissue was weighed and homogenized for 2 min in a Virtis homogenizer (if the tissue contained tough connective tissue) or a Potter-Elvejhem glass homogenizer with methanol and chloroform in the ratio 2:1:1 ($MeOH:CHCl_3$:tissue v/v/wt). As in water extractions, the chloroform phase was recovered by adding 1 volume $CHCl_3$, homogenizing for

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*The use of trade names does not imply endorsement by the National Marine Fisheries Service or the U.S. Environmental Protection Agency.

30 sec, then adding 1 volume distilled water, and homogenizing again for 30 sec. The solution was transferred to polyethlylene centrifuge tubes and centrifuged for 10 min at 5000 rpm in a Sorvall RC-5 centrifuge equipped with a SS-34 rotor. The chloroform phase was removed via a pipette, filtered through glass wool, and concentrated via rotoevaporation. All procedures were conducted at room temperature (25° C). This technique resulted in 90 to 95% recovery of chrysene.

Analysis of Extracts

Samples were analyzed using a Hewlett-Packard Model 1084A liquid chromatograph with either a Hewlett-Packard Model 1030B variable-wavelength UV detector (set at 268 nm) or a HP fixed-wavelength UV detector (254 nm). The reversed-phase columns used were either a HP RP-8 or LDC Datasorb ODS. O For most analyses, an isocratic solvent mode was effective, using $MeOH:H_2$ in the proportion 82.5:17.5% at a flow rate of 1.5 mℓ/min. For some separations, a linear gradient mode was utilized, starting with 50:50% $MeOH:H_2O$, and increasing the MeOH at the rate of 2%/min. Although chromatograms varied from animal to animal, no pre-column cleanup was necessary with the lipid extracts. A solution of 1 µg/mℓ chrysene dissolved in chloroform was used as an external standard. All samples were run in duplicate; values presented in tables and figures are averages of duplicate determinations.

Snapper

Thirty locally caught mangrove snapper, *Lutjanus griseus* (mean wet weight 163.8 g), were held in five circular (2081.9 ℓ) fiberglass tanks (1.83 m in diameter and 0.91 m high). Each tank contained 1690 ℓ natural seawater and a closed filtration system. Undergravel filters were constructed from 2.54-cm PVC pipe; seven airlift pipes were arranged to create a circular current. The filter bed consisted of crushed oyster shell. Overhead mercury vapor lamps provided 12 hr of illumination. The fish were fed frozen shrimp daily until they stopped feeding.

Before exposure, the natural disappearance rate of chrysene in the tanks was determined to maintain a constant chrysene concentration of either 1 or 5 µg/ℓ. We determined that one-half of the chrysene had disappeared within 3.5 hr after the addition of sufficient chrysene to achieve the desired concentration. After 7 to 8 hr, chrysene had virtually vanished (Figure 1). Therefore, one-half of chrysene necessary to achieve the desired initial concentration was added every 3.5 hr following initial addition of chrysene. Chrysene was applied to a tank as described; water samples were removed, extracted, and analyzed at various time intervals after the addition of contaminant. Water samples were periodically withdrawn, extracted, and analyzed during the exposure period to insure that a proper chrysene concentration was maintained.

Chrysene dissolved in acetone was added to the tanks via polyethylene tubing connected to glass syringes. The chrysene concentration of the standard in acetone delivered to 1 µg/ℓ tanks was 0.184 mg/mℓ. The 1 µg/ℓ

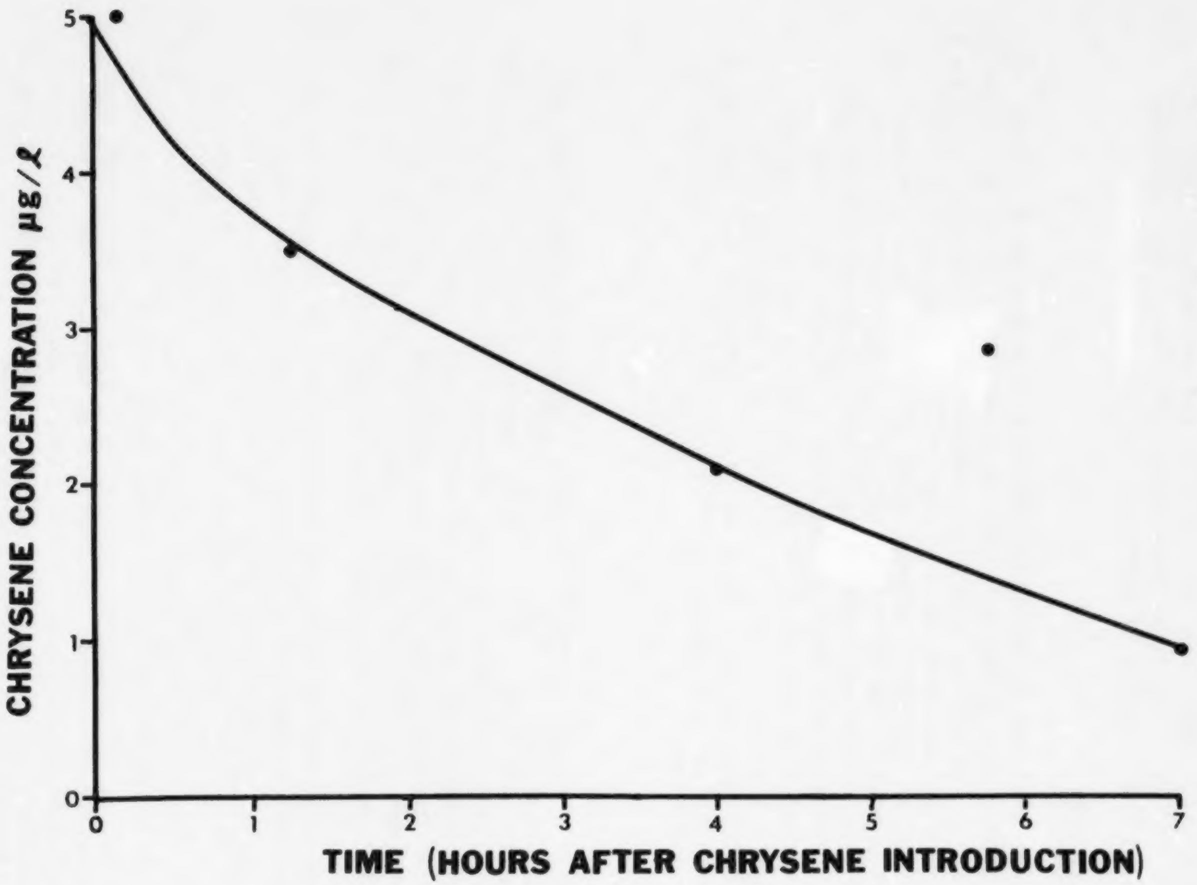

Figure 1. Decreasing concentration of chrysene as a function of time for fiberglass snapper tanks (volume = 1690 ℓ). Values are averages of duplicate samples.

tanks received 4.6 mℓ of the standard during a 15-min period every 3.5 hr. The chrysene concentration of the standard in acetone delivered to the 5 μg/ℓ tanks was 0.92 mg/mℓ. The 5 μg/ℓ tanks also received 4.6 mℓ of the standard during 15-min period every 3.5 hr. The plungers of the syringes driven by a Harvard syringe pump attached to an electric timer provided desired concent rations of the contaminant.

Chrysene was maintained at 1 and 5 μg/ℓ in two tanks for each concentration. Only one tank was used as the control. Two fish were sampled after 4 days, 7 days, and every 7 days thereafter from each tank that recieved chrysene. Four fish were sampled from the control tank after 4 days and every 14 days thereafter. One fish also was sampled from each tank before chrysene was added. Immediately after capture, fish were rinsed with chloroform, and the brain was pithed. The fish were then weighed, dissected, and filleted, and tissues (liver, gallbladder, intestine, and muscle) were analyzed.

Shrimp

At the outset of the accumulation-elimination study with pink shrimp, we determined appropriate parameters concerning the disappearance rate of chrysene in 75.8-ℓ glass aquaria, as determined for the fiberglass (snapper) tanks. Again, one-half of the chrysene disappeared after 3.5 hr and virtually vanished within 6 to 7 hr. Chrysene was added to glass aquaria in the same manner as for the fiberglass tanks to maintain constant chrysene concentrations. The chrysene concentration of the standard in acetone delivered to 1-μg/ℓ aquaria was 5.37 μg/mℓ; 1-μg/ℓ aquaria received 4.6 mℓ of the standard during a 15-min period every 3.5 hr. The chrysene concentration of the standard in acetone delivered to 5-μg/ℓ aquaria was 55 μg/mℓ. The 5-μg/ℓ aquaria received 2.22 mℓ of the standard over a 15-min period every 3.5 hr.

Chrysene dissolved in acetone was pumped by a Harvard Metering Device into 75.8-ℓ glass aquaria containing 48.7 ℓ natural seawater and maintained at 1 and 5 μg/ℓ. Four aquaria received each chrysene concentration, and two aquaria received acetone only; three aquaria served as control. Twenty pink shrimp were held in each aquarium. Filtration was under gravel with a crushed oyster-shell filter bed. The shrimp were fed daily a prepared food from the University of Arizona. The food was too small to be seen on the crushed oyster shell; therefore, the amount was increased or decreased according to the appearance of the water (yellowing of the water indicated leaching of food). The number of molts was recorded daily.

Shrimp were sampled from each aquarium containing both concentrations at Day 4, 7, and every 7 days thereafter. Water samples were withdrawn periodically to check contaminant levels. After chrysene contamination was discontinued, each aquarium was drained, rinsed, and refilled with clean seawater three times. Shrimp were sampled from the four aquaria that received chrysene at the same intervals as during accumulation. Accumulation and elimination were both measured for 28 days. Shrimp were sampled from each control aquarium at Day 0, 4, 14, and 28 during both accumulation and elimination periods. No shrimp were sampled from acetone control aquaria (maintained only during accumulation period). Immediately after removal from the aquaria, the shrimp were washed with seawater and frozen. Since the exoskeleton was removed, the shrimp were not rinsed in chloroform. Before analysis, each sample was thawed and dissected. Tissues of the cephalothorax, including the appendages, abdomen, and intestine (when 0.2 g was available) were analyzed. The carapace was removed from the cephalothorax and the exoskeleton, telson, and uropods removed from the abdomen before extraction.

RESULTS

Snapper

This experiment was terminated prematurely when snapper died in all tanks, including those in the control on Day 20. Deaths of the fish began 5 days after exposure to 1 and 5 μg/ℓ chrysene and after the 13th day in the control tank. No difference in mortality was noted in fish exposed to 1 or 5 μg/ℓ chrysene.

There appeared to be little difference in total food consumption by fish exposed to the two chrysene concentrations either at the end of the experiment and before the mass mortalities. On Day 4, food consumption by fish declined in all tanks except the control, where a higher consumption level was observed for the remainder of the experiment.

Tables 1 and 2 summarize results of 20-day exposure of *Lutjanus griseus* to chrysene. After exposure to both 1 and 5 μg/ℓ chrysene, *L. griseus* concentrated the PAH in the liver (Figure 2), but no consistent significant accumulation was observed in other tissues studied. After 20 days, fish exposed to 5 μg/ℓ chrysene accumulated 1300 μg/kg of the PAH (260 times the exposure level); fish exposed to 1 μg/ℓ accumulated 360 μg/kg chrysene in their liver (360 times the exposure concentration). Livers of fish that received 1 μg/ℓ chrysene accumulated 27% of the amount of chrysene that was accumulated in the livers of fish exposed to the 5 μg/ℓ chrysene.

Shrimp

Shrimp exposed to both 1 and 5 μg/ℓ chrysene accumulated the PAH in the cephalothorax and abdomen (Tables 3, 4); no chrysene was detected in the intestines, nor was any significant amount detected in the control organisms.

More chrysene was concentrated in the cephalothorax than in the abdomen at both 1 and 5 μg/ℓ concentrations. Shrimp exposed to 5 μg/ℓ chrysene concentrated the PAH approximately 360-fold in the cephalothorax, and approximately 84-fold accumulated in the abdomen by the end of the exposure period (28 days) (Figure 3). Chrysene accumulated in the cephalothorax throughout the exposure period, whereas concentrations in the abdomen remained fairly constant after 14 days. The cephalothorax of

TABLE 1. ACCUMULATION OF CHRYSENE IN VARIOUS TISSUES AFTER MANGROVE SNAPPER, *LUTJANUSGRISEUS*, WERE EXPOSED TO A 1μg/ℓ CONCENTRATION IN A CLOSED SEAWATER SYSTEM[*]

	DAYS EXPOSURE				
Tissue	0	4	7	14	20
White Muscle	n.d.[**]	n.d.	n.d.	n.d.	17.4
Liver	n.d.	104	105	308	367
Intestine	n.d.	n.d.	n.d.	n.d.	190
Gallbladder	n.d.	n.d.	n.d.	n.d.	n.d.

[*] Values given are in μg/kg. Wet weight basis.

[**] none detected which indicates amounts $\leq 5 \times 10^{-9}$ g/injection

TABLE 2. ACCUMULATION OF CHYRYSENE IN VARIOUS TISSUES AFTER MANGROVE SNAPPER, *LUTJANUS GRISEUS*, WERE EXPOSED TO A 5 μg/ℓ CONCENTRATION IN A CLOSED SEAWATER SYSTEM[*]

	DAYS EXPOSURE				
Tissue	0	4	7	14	20
White Muscle	n.d.[**]	15.8	n.d.	n.d.	n.d.
Liver	n.d.	415	406	810	1,294
Intestine	n.d.	n.d.	n.d.	n.d.	n.d.
Gallbladder	n.d.	n.d.	n.d.	n.d.	n.d.

[*] Values given are in μg/kg. Wet weight basis.

[**] none detected

TABLE 3. ACCUMULATION AND ELIMINATION OF CHRYSENE IN VARIOUS TISSUES OF *PENAEUS DUORARUM* EXPOSED TO A 1 μg/ℓ CONCENTRATION IN A CLOSED SEAWATER SYSTEM*

	DAYS EXPOSURE										
	ACCUMULATION						ELIMINATION				
Tissue	0	4	7	14	21	28	33	35	42	49	56
Cephalothorax	n.d.**	84	170	251	140	248	229	196	151	89	48
Abdomen	25	91	124	188	188	199	155	142	91	90	91
Intestine	n.d.	n.d.	-	n.d.	-	n.d.	-	-	-	-	-

* Values given are in μg/kg. Wet weight basis.
** none detected.

TABLE 4. ACCUMULATION AND ELIMINATION OF CHRYSENE IN VARIOUS TISSUES OF *PENAEUS DUORARUM* EXPOSED TO A 5 μg/ℓ CONCENTRATION IN A CLOSED SEAWATER SYSTEM*

	DAYS EXPOSURE										
	ACCUMULATION						ELIMINATION				
Tissue	0	4	7	14	21	28	33	35	42	49	56
Cephalothorax	n.d.**	476	450	634	626	1,809	525	418	190	536	105
Abdomen	25	260	199	423	197	418	218	216	148	104	112
Intestine	n.d.	n.d.	n.d.	-	-	-	-	-	-	-	-

* Values given are in μg/kg. Wet weight basis.
** none detected.

shrimp exposed to 1 μg/ℓ levels accumulated chrysene by approximately 250-fold over exposure levels, while the abdomen concentrated chrysene by approximately 200-fold (Figure 4).

By the end of the experiment pink shrimp exposed to 5 μg/ℓ chrysene had accumulated approximately 4.3 times more chrysene in the cephalothorax than in the abdomen; pink shrimp exposed to 1 μg/ℓ chrysene accumulated approximately 1.2 times the amount of chrysene in the cephalothorax than in the abdomen.

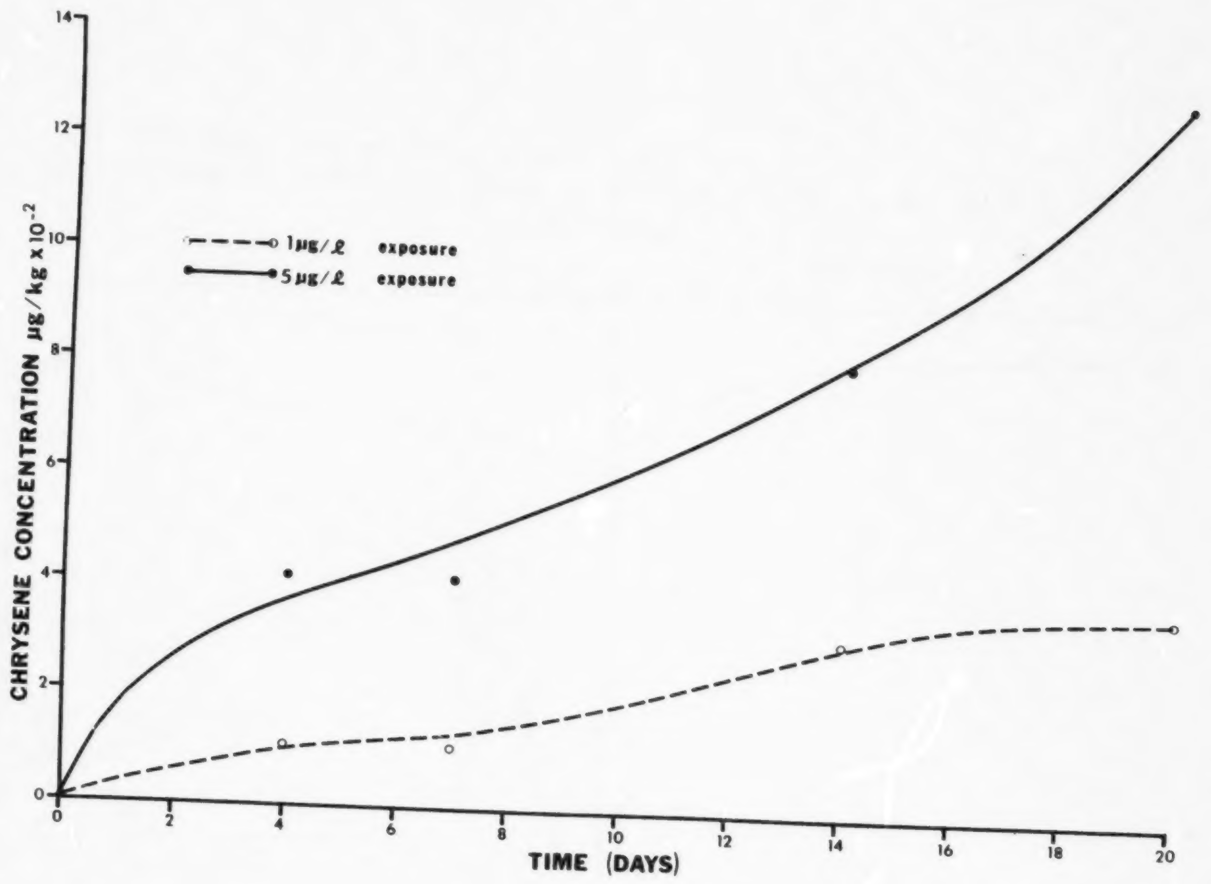

Figure 2. Accumulation of chrysene in mangrove snapper, *Lutjanus griseus*, liver tissue.

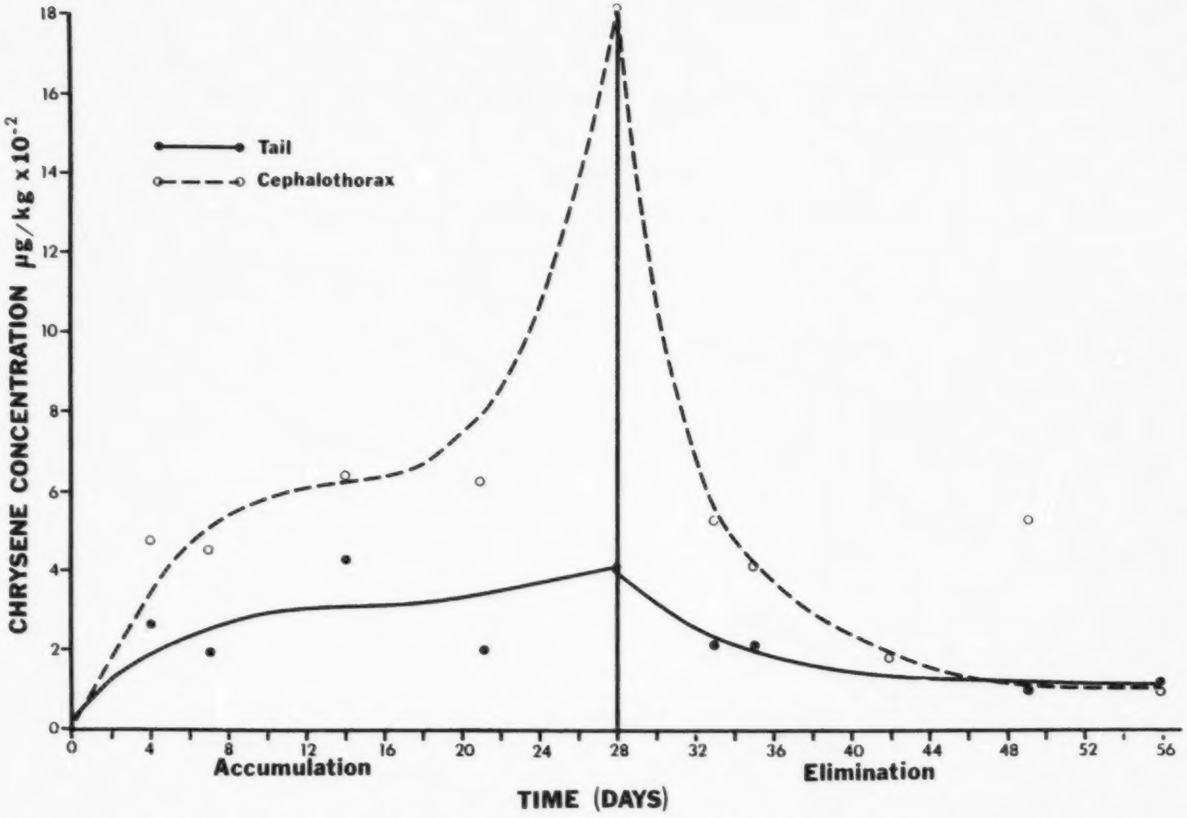

Figure 3. Accumulation and elimination of chrysene by pink shrimp, Penaeus duorarum, exposed to the 5 µg/ℓ level.

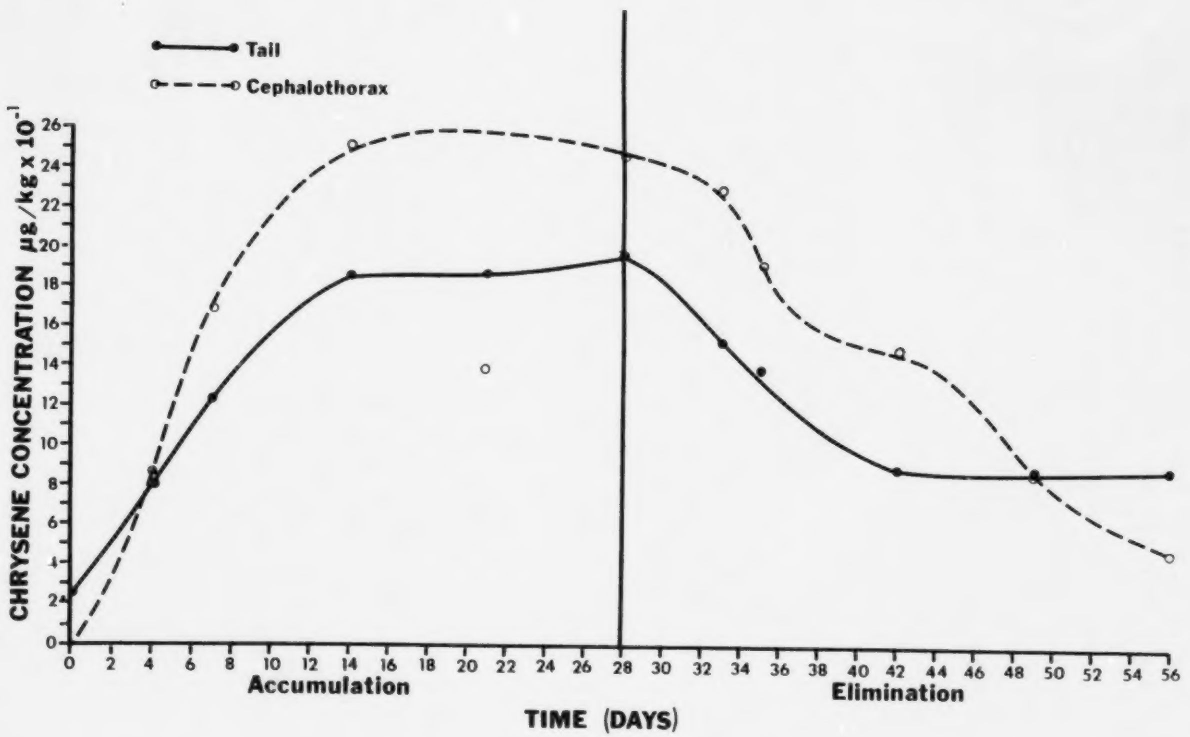

Figure 4. Accumulation and elimination of chrysene by pink shrimp, Penaeus duorarum, exposed to the 1 µg/ℓ level.

The cephalothorax of shrimp exposed to 5 μg/ℓ chrysene contained approximatly 7.3 times more of the PAH than the cephalothorax of shrimp exposed to 1 μg/ℓ chrysene. Abdomens of shrimp exposed to 5 μg/ℓ chrysene incorporated approximately 2.1 times as much chrysene as those of shrimp exposed to 1 μg/ℓ chrysene.

When the shrimp were returned to chrysene-free water, an initial rapid loss of chrysene occurred in both body sections, followed by a longer phase of gradual release. Release was more rapid in the cephalothorax than in the abdomen, and the most rapid in cephalothorax from shrimp exposed to 5 μg/ℓ chrysene. There was little difference in food consumption between shrimp receiving either chrysene concentration or acetone without chrysene. Control shrimp consumed slightly more food than shrimp receiving chrysene. Frequency of molting was approximately equal among shrimp during the 28-day exposure to both chrysene concentrations or acetone without chrysene, and shrimp used as controls. Molting during the following 28 days, however, was more frequent in shrimp at both chrysene concentrations than in control shrimp.

During the accumulation period mortality was higher in the aquaria that received both concentrations of chrysene and acetone only than the control aquaria. No difference in mortality was noted among shrimp at both concentrations of chrysene. With the exception of one aquarium, mortality began occurring on the 13th day of the chrysene exposure. From 1 to 9 shrimp were found dead each day for the remaining 15 days in the aquaria receiving chrysene. Three deaths occurred in the control aquaria after air lines had become disconnected. During the elimination period there were only two deaths in the aquaria that received chrysene and none in the control aquaria. There was no visible evidence of disease in any of the aquaria; however, no histological examinations were made.

DISCUSSION

The results of our study indicate that the two organisms studied, mangrove snapper and pink shrimp, can rapidly accumulate chrysene in their tissues. In mangrove snapper, chrysene accumulated in significant amounts only in the liver after 20-day exposure. In contrast to the findings of other researchers (Neff et al., 1976), snapper continued to concentrate the aromatic hydrocarbon after 20 days. Other researchers have found that aromatic hydrocarbons accumulate in the livers of other fish species (Lee et al., 1972) and therefore are transferred to the gallbladder. Although no chrysene was detected in the gallbladder in our study, it is possible that its metabolites, which were not monitored, could have been present. Pink shrimp accumulated chrysene in the cephalothorax and abdomen with increasing concentration as a function of time.

Difficulty in preventing disease in fish held in a closed laboratory system was encountered in tests using the chrysene/acetone mixture. We observed that when acetone was added to tanks, shrimp suffered a severe sloughing of the mucous membrane. Sloughing probably also occurs in similar tests with fish, increasing their susceptibility to disease.

The first appearance of disease in the snapper tanks occurred only 5 days after the chrysene/acetone mixture was added. At that time, foam was prevalent on the water's surface. On the following day, the first two mortalities occurred. The water had a slimy appearance that remained throughout the experiment.

Airstones were frequently clogged and had to be changed. By Day 10, the majority of fish in one tank had cloudy eyes, and on the following day, 11 died. (The cause was diagnosed as Cryptocaryon.) Also on Day 11, the copepod, Argulus, was visible in each tank and on the fish, and was more numerous in the tanks receiving chrysene than in the control tank. The water was changed in all tanks and 0.25 μg/ℓ dylox added. No copepods were visible in the water or on the fish the following day or thereafter. Water was changed again that day because some fish in every tank had cloudy eyes; 25 μg/ℓ formaldehyde was also added to the tanks that received chrysene.

Shrimp exposed to either 1 or 5 μg/ℓ chrysene concentrated more of the contaminant in the cephalothorax than in the abdomen, especially shrimp exposed to 5 μg/ℓ chrysene. Other researchers, using different aromatics, have found increased retention in the cephalothorax, especially in the digestive gland of brown shrimp, Penaeus aztecus (Neff et al., 1976). This organ may act as a storage place for lipids (Vonk, 1969). After chrysene exposures ceased, an initial rapid decrease in amounts of contaminant occurred, followed by a prolonged slower release. Perhaps the initial phase represents active excretion, while the second phase represents passive diffusion. Whatever the mechanism of release, it should be noted that detectable levels of the carcinogen were present even 28 days after termination of exposure to 1 μg/ℓ concentration of chrysene.

Aquaria that received the chrysene/acetone mixtures and acetone only had a large buildup of slime in the air tubes and on the inner sides. The air tubes and stones frequently clogged and had to be cleaned. This reduction in air was probably responsible for some deaths. During the last 8 days of the tests, water in the aquaria that received the chrysene/acetone mixtures and acetone without chrysene became so cloudy that three water changes were required. The rapid and extensive production of foam was also evident on the water's surface. This excessive production of slime is probably due to a sloughing of the mucous membranes in the presence of acetone. There was no slime buildup or foam in the control aquaria.

Our study demonstrates that mangrove snappers and pink shrimp accumulate chrysene after prolonged exposures. Although mangrove snappers accumulate chrysene in their livers, liver tissue is not used for human consumption and does not pose a health hazard to humans at this time. Pink shrimp, on the other hand, accumulated chrysene in the cephalothorax and abdomen (tail). Shrimp exposed to 5 μg/ℓ chrysene concentrated the hydrocarbon by 360-fold in the cephalothorax as compared to approximately 84-fold in the tail. Initially pink shrimp were able to eliminate chrysene very rapidly from the cephalothorax and tail after exposure to the contaminant was terminated. After the rapid initial elimination, a rather slow elimination process was observed. The shrimp still contained large amounts of chrysene in both the cephalothorax and tail after 28 days in chrysene-free seawater.

In our opinion, the level of chrysene remaining in the shrimp after 28 days of elimination could pose a serious health hazard if these shrimp were consumed by humans for an extended period of time. Our primary consideration at this point should be a more intensive study to project the probable concentrations and duration of exposure of these two organisms to chrysene during a crude-oil or shale-oil spill in the natural environment. The data derived from a study of this scope would be extremely valuable in determining whether an oil spill would actually contaminate commercially important marine species used for human consumption with carcinogens of sufficient quantity to pose a serious human health hazard.

ACKNOWLEDGMENTS

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ACCUMULATION, TISSUE DISTRIBUTION, AND DEPURATION OF BENZO(a)PYRENE AND BENZ(a)ANTHRACENE IN THE GRASS SHRIMP, PALAEMONETES PUGIO

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ABSTRACT

The short-term uptake, tissue distribution, and depuration of two polycyclic aromatic hydrocarbons, ^{14}C-benzo(a)pyrene (BP) and ^{14}C-benz(a)anthracene (BA), were studied utilizing the grass shrimp, Palaemonetes pugio, at known stages of the molt cycle. Premolt shrimp accumulated less BP and BA than intermolt shrimp. The newly molted shrimp accumulated more BA than intermolt shrimp. At each of the concentrations tested [1.25, 2.5, 5.0, 10.0 parts per billion(ppb)], intermolt shrimp accumulated BA to a greater extent than BP. The BA or BP accumulated by shrimp increased in relation to environmental levels of these compounds. The accumulation of BP and BA in tissues examined was in the following order: digestive tract (stomach + intestine)> hepatopancreas> cephalothorax> abdomen. All tissues accumulated more BA than BP. When exposed to media containing 2.5 ppb BP or 2.8 ppb BA, a rapid uptake by shrimp was noted during the first 6-hr exposure, subsequently uptake was reduced for BP. However, at termination of 96-hr exposure, shrimp exhibited a trend of continual accumulation of BA and BP. When transferred to seawater, shrimp appeared to depurate BA more rapidly than BP. In the shrimp exposed to BA, the level of radioactivity declined by 80% after a 7-day depuration; under similar conditions, the BP level (radioactivity) declined by only 35%.

INTRODUCTION

Concern with the possible contamination of the aquatic environment by polycyclic aromatic hydrocarbons (PAHs) has led to an increase in studies of the effects of these compounds on marine animals. Although PAHs are derived from airborne particulates produced by forest fires, refuse burning and the combustion of fossil fuels, petroleum and its products are also implicated in PAH contamination. The detection of PAH in marine fish

(Pancirov and Brown, 1977) and shellfish (Cahnmann and Karatsune, 1957; Erhardt, 1972; Dunn and Stich, 1976; Bravo *et al.*, 1978;) collected from polluted waters adds relevance to the investigations of PAH contamination of the marine ecosystem.

The accumulation, distribution, and release of PAH have been investigated in commercially important shellfish (Lee *et al.*, 1972; Neff and Anderson, 1975; Dunn and Stich, 1976) that are raised for human consumption. The clam, *Rangia cuneata*, took up approximately 200 times the ambient level of benzopyrene (BP) and retained about twice the ambient level of BP at the end of thirty hours (Neff and Anderson, 1975).

Studies of PAH in crustaceans have been limited to copepods (Lee, 1975) and the blue crab, *Callinectes sapidus* (Lee *et al.*, 1976). In both studies it was found that crustaceans rapidly accumulate BP, reaching a maximum in 2 days, whereas the release of BP is slow, occurring over two to three weeks. Studies using the blue crab examined the distribution of BP and its metabolites in various tissues.

In studies with crustaceans, the stage of the molt cycle must be considered. Changes in cuticle permeability occur in relation to cyclic shedding, secretion, and hardening of the exoskeleton in crustaceans (Passano, 1960; Conklin and Rao, 1978). Conklin and Rao (1978) showed that the uptake of pentachlorophenol, a chlorinated aromatic hydrocarbon, varied with the stage of the molt cycle, the greatest accumulation occurring immediately after ecdysis. Our paper discusses the accumulation, tissue distribution, and retention of the polycyclic aromatic hydrocarbons, benzopyrene and benzanthracene, at various stages of the molt cycle.

Materials and Methods

Animals--Grass shrimp, *Palaemonetes pugio*, were collected from grass beds in Santa Rosa Sound, Gulf Breeze, FL, and maintained in large aquaria containing filtered (5 μm) seawater of 10 ^{o}/oo salinity. Shrimp were used within three weeks of collection and were not fed during experiments.

Experimental design--Intermolt shrimp were exposed to 1.25, 2.5, 5.0, 10.0 ppb of either [7, 10-^{14}C]-benzo(a)pyrene (60.7 mCi/mmole) or [12-^{14}C]-benz(a)anthracene (49 mCi/mmole) in 10 ^{o}/oo seawater (200 mℓ/animal) animal). At the end of 6-, 12-, and 24-hr exposures, shrimp were transferred to PAH-free seawater for a 2-min wash. Animals were blotted dry, weighed, and placed into Protosol tissue solubilizer. Digested samples were neutralized with acetic acid, and 15mℓ of Aquasol II were added. Samples were counted in a Beckman LS-133 liquid scintillation counter, and values were corrected for quench and machine efficiency. A sample (0.5 mℓ) of the medium was taken for each isotope to determine the exposure levels of each isotope at the beginning of the experiment.

Animals at various stages of the molt cycle were exposed to a medium (400 mℓ/shrimp) of 10 ^{o}/oo seawater containing either 2.5 ppb (2.5 μg/ℓ) benzopyrene (BP) or 2.8 ppb benzanthracene (BA) for 3 hr to test the effect of ecdysis on accumulation. The molt cycle stage was determined by the method of Conklin and Rao (1978). After 3 hr, shrimp were placed in clean seawater for two min, blotted dry, weighed, and placed in tissue solubilizer.

Grass shrimp in stage C (intermolt stage of the molt cycle) were used to determine the accumulation, retention, and distribution of BP and BA. In the accumulation experiment, shrimp were exposed from 15 min to 96 hr in either 2.5 ppb BP or 2.8 ppb BA. Animals were washed in clean seawater, dried, weighed, and solubilized before scintillant was added; samples were counted for radioactivity.

Tissue distribution of BP and BA was followed during the first 24 hr of accumulation. At the appropriate time intervals, shrimp were washed and dried before dissection to remove the hepatopancreas, the digestive tract (stomach and intestine), and the abdominal muscle. The remainder of the shrimp (cephalothorax) as well as the tissues dissected were solubilized and counted for radioactivity after being weighed. Shrimp were exposed to either 2.5 ppb BP or 2.8 ppb BA for 12 hr. They were then transferred to uncontaminated seawater and sacrificed at intervals ranging from 3 to 168 hr for analysis of radioactivity. The tissue distribution of radioactivity from BP or BA was observed during the depuration process. The four parts of the shrimp discussed above were analyzed for retention of radioactivity.

Intermolt shrimp and newly molted shrimp were exposed to BP or BA for 3 hr to compare tissue distribution. The four parts of the dissected shrimp were analyzed for radioactivity.

Chemicals--^{14}C-Benzopyrene and ^{14}C-benzanthracene were purchased from Amersham/Searle, Arlington Heights, IL. Protosol and Aquasol II were purchased from New England Nuclear Corp., Boston, MA.

Results

The accumulation of [7, 10-^{14}C]-benzo(a)pyrene in grass shrimp exposed to 1.25, 2.5, 5.0, and 10.0 ppb of benzopyrene (BP) medium is shown in Figure 1. Uptake in shrimp exposed to 10.0 ppb was approximately 5 times as great as that of shrimp exposed to 1.25 ppb at 12 and 24 hr exposure. The difference was much less at 6 hr exposure. Shrimp exposed to 2.5 ppb accumulated about 1.5 times as much as shrimp in 1.25 ppb, while shrimp in 5.0 ppb medium took up about 2.5 times that of shrimp in the least concentrated medium.

The accumulation of [12-^{14}C]-benz(a)anthracene in shrimp exposed to the four concentrations is shown in Figure 2. Shrimp exposed to 10.0 ppb benzanthracene (BA) medium had an uptake about 8 times that of shrimp exposed to 1.25 ppb medium when exposed for 12 and 24 hr; those in 2.5 ppb took up almost twice the mount of shrimp exposed to 1.25 ppb. The difference was less after 6 hr exposure. The shrimp in 5.0 ppb BA accumulated about 4 times as much as those in 1.25 ppb medium.

To conserve labeled compounds yet maintain sufficient levels of radioactivity, we used 2.5 ppb BP or 2.8 ppb BA (equivalent in DPM to 2.5 ppb BP) for the remaining experiments.

Accumulation of BP and BA by the grass shrimp, Palaemonetes pugio, in different molt stages is shown in Figure 3. Although BP uptake varies considerably in the stages of the molt cycle, BA was taken up more than BP at every stage of the molt cycle (the greatest difference was observed immediately after the molt).

Shrimp were exposed to experimental media for various times to observe the accumulation rate. As shown in Figure 4, grass shrimp accumulated benzanthracene to a greater extent than benzopyrene for the time intervals tested. Both compounds were taken up quickly during the first 6 hr. During the next 3 days, BA continued to accumulate while BP appeared to maintain the same level of incorporation.

Accumulation of benzopyrene in the tissues paralleled that observed in the whole body (Figure 5). The amount increased in all the tissues in the first 3 hr but changed relatively little during the next 18 hr. The digestive tract (stomach and intestine) showed the greatest uptake at 6 hr while hepatopancreas had about one-fourth the amount observed in the digestive tract. The abdominal muscle and cephalothorax were relatively low in radioactivity.

Benzanthracene uptake in tissues also paralleled the whole animal trend. Figure 6 shows that only the abdominal muscle failed to show an increase in uptake after 1 hr exposure. The digestive tract had the greatest accumulation over the 24-hr period. The hepatopancreas accumulated about one-half and the abdominal muscle and the cephalothorax about one-twentieth of that found in the digestive tract.

The distribution of BP and BA in the four parts of the intermolt and newly molted shrimp was examined after a 3 hr exposure (Table 1). The accumulation of BP in the tissue other than the abdominal muscle of newly molted shrimp was not significantly different from that in intermolt shrimp. But a nearly three-fold increase in uptake of BP occurred in the abdominal muscle of newly molted shrimp in comparison with intermolt shrimp. The accumulation of BA in the abdominal muscle, hepatopancreas, and digestive tract of newly molted shrimp was significantly ($P = 0.05$) different from that in intermolt shrimp. A nearly two-fold increase was observed for these tissues.

Figure 7 shows the retention of BP and BA radioactivity after an exposure period of 12 hr. Benzanthracene was lost from grass shrimp faster and to a greater extent over the seven day depuration period than benzopyrene; 35% of the accumulated BP radioactivity was lost by the shrimp after 7 days, while about 80% of the initial BA radioactivity was lost from shrimp during the same period.

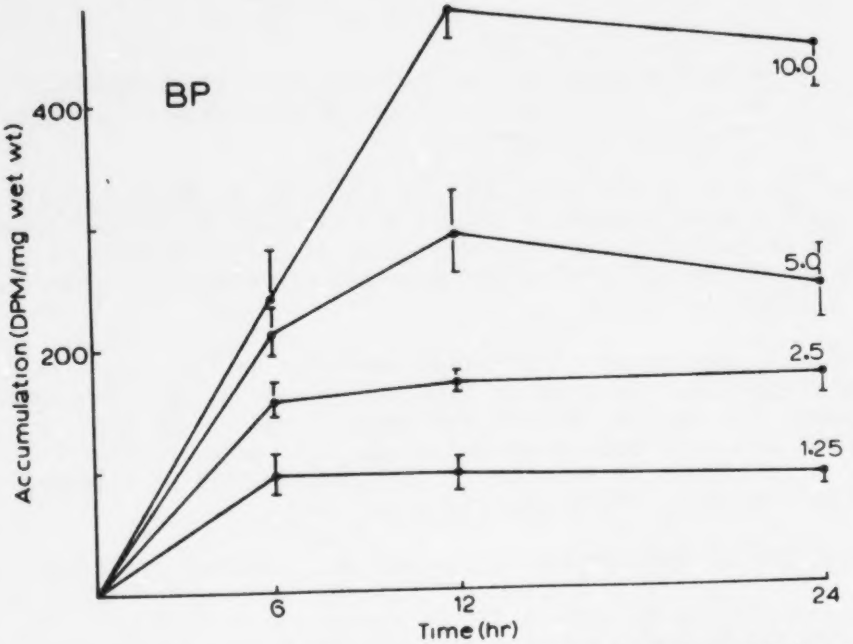

Figure 1. Accumulation of benzopyrene in stage C shrimp which were exposed to four concentrations (ppb) of the hydrocarbon for three time intervals. The medium (200 mℓ/shrimp) contained 17 DPM/ℓ. Values are the mean ± SEM for 8 shrimp.

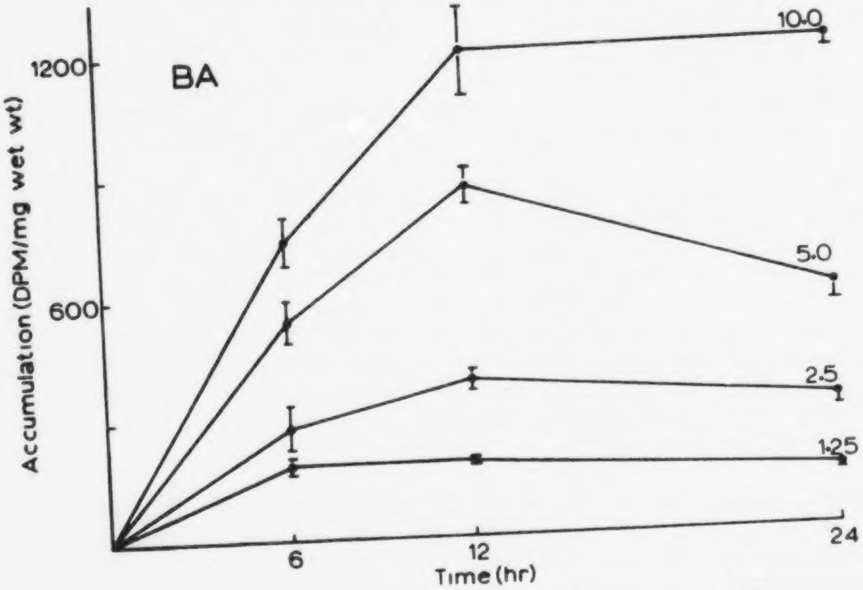

Figure 2. Uptake of benzanthracene in stage C shrimp which were exposed to four concentrations (ppb) of the hydrocarbon for three time periods. The medium (200 mℓ/shrimp) contained 17.3 DPM/µℓ. Values are the mean ± SEM for 8 shrimp.

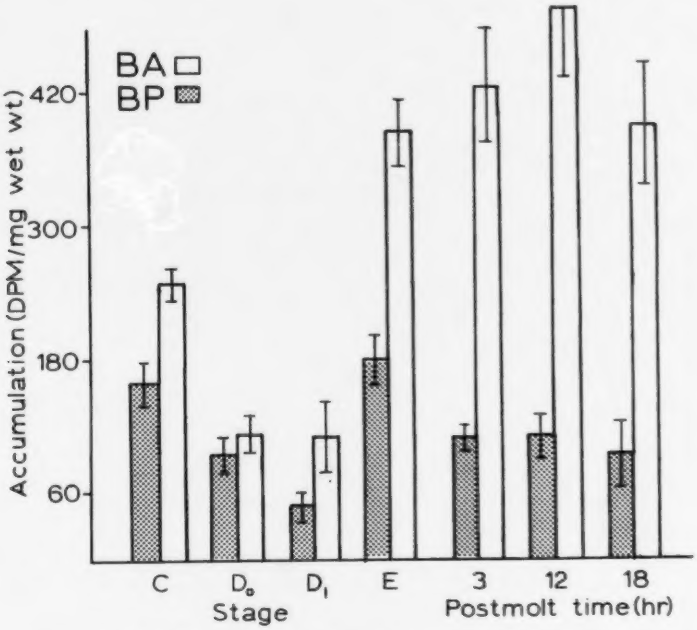

Figure 3. Accumulation of benzopyrene and benzanthracene at various stagesp) of the molt cycle in the grass shrimp. The medium (400 mℓ/shrim contained 17 DPM/μℓ or 17.3 DPM/μℓ, respectively. Shrimp remained in the medium for 3 hr. C = intermolt, D = premolt, E = ecdysis (molt). Values are the mean ± SEM for 8 shrimp.

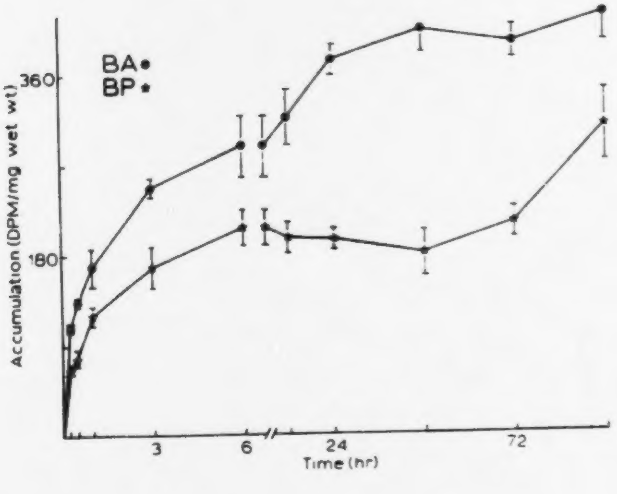

Figure 4. Accumulation of benzopyrene and benzanthracene over 96-hr period in stage C shrimp. The medium contained 2.5 ppb BP or 2.8 ppb BA (200 mℓ/shrimp) which had 17 DPM/μℓ or 17.3 DPM/μℓ, respectively. The values are the mean ± SEM for 8 shrimp.

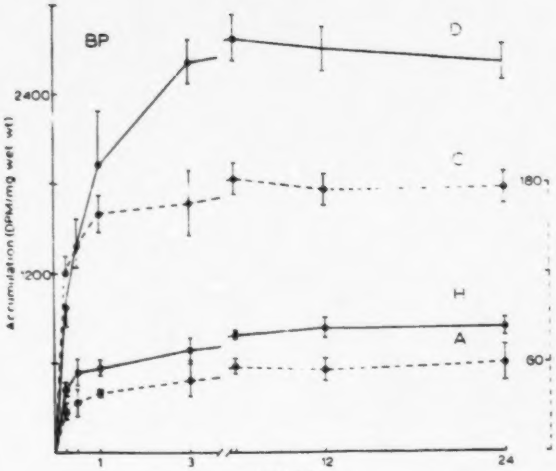

Figure 5. Uptake of benzopyrene (2.5 ppb) in the tissues of the grass shrimp in 24 hr. The medium (200 mℓ/shrimp) contained 17 DPM/μℓ. A=abdominal muscle, C=cephalothorax, D=digestive tract, H=hepatopancreas. Values are given as the mean ± SEM for 7 shrimp.

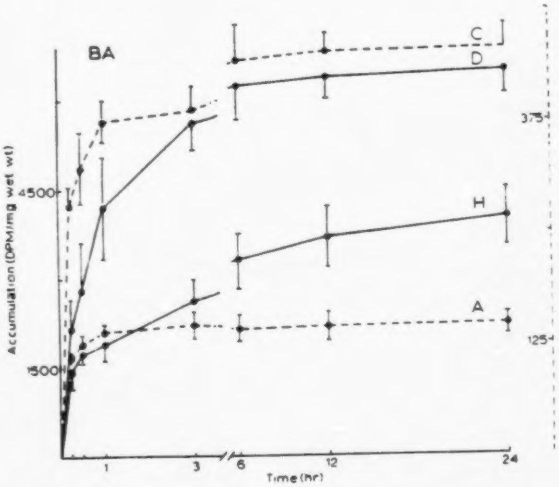

Figure 6. Uptake of benzanthracene (2.8 ppb) in the tissues of the stage C grass shrimp in 24 hr. The medium (200 mℓ/shrimp) contained 17.3 DPM/μℓ. A=abdominal muscle, C=cephalothorax, D=digestive tract, H=hepatopancreas. Values are the mean ± SEM for 8 shrimp.

TABLE 1. DISTRIBUTION OF PAH IN TH TISSUES OF INTERMOLT AND ECDYSIAL GRASS SHRIMP

Tissues	Benzopyrene Uptake (DPM/mg wet wt.)		Benzanthracene Uptake (DPM/mg wet wt.)	
	Intermolt	Molt	Intermolt	Molt
Digestive tract (stomach + intestine)	2604 ± 162 (7)	3028 ± 355 (8)	5409 ± 462 (8)	8190 ± 620 (7)
Hepatopancreas	694 ± 78 (7)	1089 ± 182 (7)	2528 ± 363 (8)	4838 ± 620 (7)
Cephalothorax	166 ± 23 (7)	207 ± 22 (7)	387 ± 29 (8)	479 ± 53 (6)
Abdominal muscle	49 ± 10 (8)	131 ± 20 (7)	143 ± 16 (7)	327 ± 41 (7)

Shrimp were exposed to either 2.5 ppb benzopyrene (17 DPM/$\mu\ell$) or 2.8 ppb benzanthracene (17.3 DPM/$\mu\ell$) for 3 hr. After transfer to seawater, the shrimp were dissected for the tissues which were weighed, solubilized and counted in a liquid scintillation counter. The values are expressed as the mean ± SEM and the number of animals are given in parenthesis. A t-test was done on the intermolt and molt values for each compound. BP values for the abdominal muscle were significantly different at the 0.05 level. The BA values for digestive tract, hepatopancreas and abdominal muscle are significantly different at the 0.05 level. Other values are not significant.

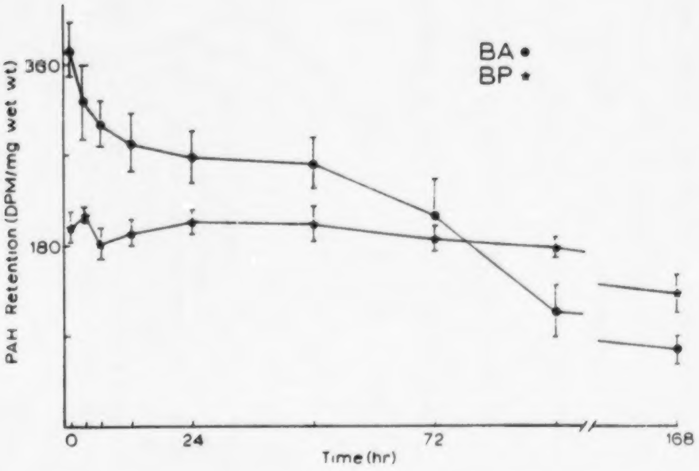

Figure 7. Retention of benzopyrene (2.5 ppb) and benzanthracene (2.8 ppb) in the stage C shrimp during a 7-day depuration period. The shrimp were exposed to the PAH (200 mℓ/shrimp) for 12 hr before transfer to seawater. The medium contained 17 DPM/µℓ or 17.3 DPM/µℓ, respectively. Values are the mean ± SEM for 7 shrimp.

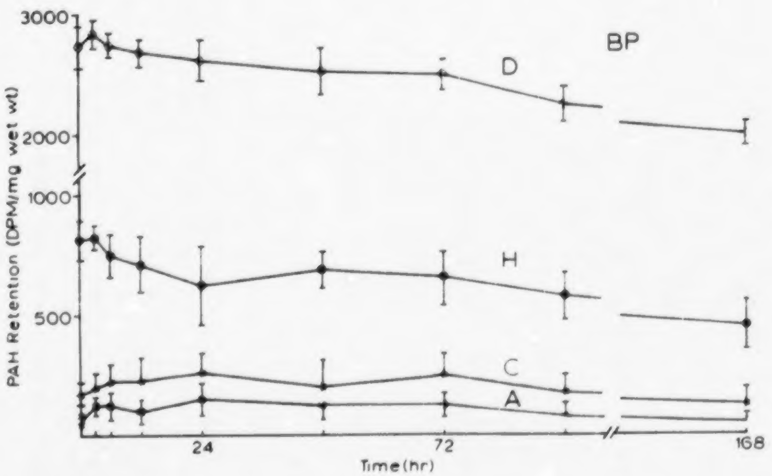

Figure 8. Retention of benzopyrene (2.5 ppb) in the tissues of the shrimp during a 7-day depuration. The shrimp were exposed to the PAH (200 mℓ/shrimp) for 12 hr prior to removal to uncontaminated seawater. The medium contained 17 DPM/µℓ. A=abdominal muscle, C=cephalothorax, D=digestive tract, H=hepatopancreas. Values are the mean ± SEM for 6 shrimp.

The retention of benzopyrene radioactivity by the different tissues is shown in Figure 8. Although the four parts of the shrimp showed little reduction in radioactivity, the digestive tract decreased appreciably over the 7-day period. These results agreed with the whole-body counts.

The pattern of depuration of benzanthracene radioactivity in the grass shrimp tissues (Figure 9) is consistent with that of whole animals. The abdominal muscle showed the least change, whereas digestive tract again had the greatest loss of radioactivity. The hepatopancreas and the cephalothorax lost very little radioactivity until the fourth day.

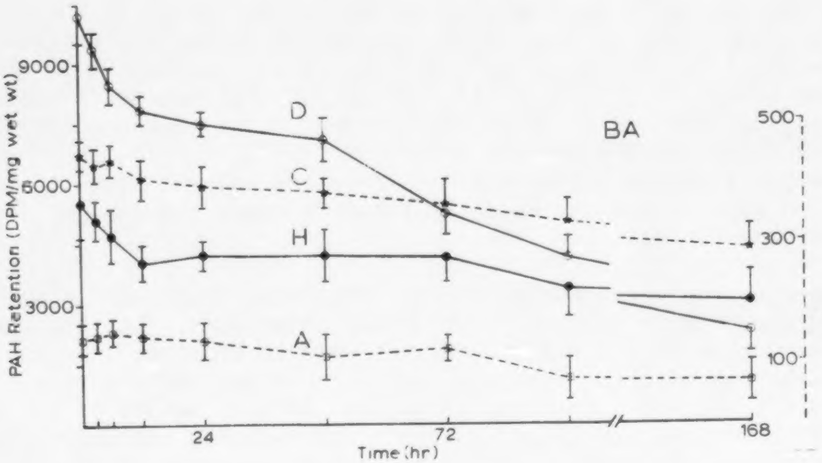

Figure 9. Retention of benzanthracene (2.8 ppb) in the tissues of the shrimp during a 7-day depuration . The shrimp were exposed to the compound (200 mℓ/shrimp) for 12 hr prior to removal to clean seawater. The medium contained 17.3 DPM/μℓ. A=abdominal muscle, C=cephalothorax, D=digestive tract, H=hepatopancreas. Values are the mean ± SEM for 7 shrimp.

DISCUSSION

The accumulation of aromatic hydrocarbons by a variety of aquatic animals has been studied (Lee *et al*., 1972a,b, 1976; Stegeman and Teal, 1973; Lee, 1975; Neff and Anderson, 1975; Neff *et al*., 1976; Harris *et al*., 1977; Rossi and Anderson, 1977; Roubal *et al*., 1977; Melancon and Lech, 1978). Accumulation is rapid in fish (Melancon and Lech, 1978) as well as in crabs (Lee *et al*., 1976) and oysters (Neff *et al*., 1976). Most of the compounds reach a maximum concentration in one to two days; however, depuration generally occurs over a 3-week period.

The uptake of PAH in grass shrimp was rapid as observed in previous studies of aromatic hydrocarbon uptake in crustaceans. However, the influence of the molt cycle showed considerable variation in uptake of the hydrocarbons. Our studies indicate that PAHs may enter the shrimp more easily at ecdysis when the cuticle is more permeable. Conklin and Rao (1978) presented evidence that the grass shrimp accumulates pentachlorophenol to a greater extent at ecdysis than at any other stage in the molt cycle.

The incorporation of radiolabeled benzopyrene and benzanthracene into shrimp within minutes of exposure to these compounds has shown the permeabililty of crustaceans to these PAH. Lee and others (1972b) detected large amounts of benzopyrene in tissues of marine fish within minutes of exposure. The uptake of naphthalenes occurs within the first 10 hr of exposure in clams (Neff et al., 1976), within the first 3 hr exposure in marine polychaete worms (Rossi and Anderson, 1977), and within 30 min in brown shrimp (Anderson et al., 1974), indicating that marine invertebrates are rapid accumulators of aromatic hydrocarbons. The bioaccumulation of BP in clams was 200 times the ambient level (Neff et al., 1976) in 24 hr, whereas the grass shrimp accumulated 18 times the ambient level at the end of 96 hr. However, grass shrimp accumulated 24 times the ambient level of BA over the same period.

Benzopyrene and benzanthracene distribution in grass shrimp was not in total agreement with studies on other marine animals. Lee and coworkers (1972b) showed that fish liver accumulates more benzopyrene than stomach and that crab hepatopancreas took up considerably more benzopyrene than the stomach (1976). However, in terms of uptake per unit weight of the tissue, the stomach (digestive tract) accumulated more PAH than hepatopancreas in the grass shrimp. The abdominal muscles in the crab (Lee et al., 1976) and brown shrimp (Neff et al., 1976) appeared to take up as much hydrocarbon as the stomach; yet the grass shrimp muscle accumulated very low quantities compared to digestive tract on a tissue weight basis. The digestive tract accumulated BP 154 times the ambient level, while BA accumulation was 376 times the ambient level. The muscle and the cephalothorax accumulated the same or less than the whole shrimp: muscle had 8.5 times the BA and 3.5 times the BP; the cephalothorax, 25 times the BA and 10 times the BP. The hepatopancreas accumulated almost 5 times as much BA (bioaccumulation factor = 231) than BP (bioaccumulation factor = 49).

In studies of the distribution of BP and BA in the tissues of intermolt and newly molted shrimp, BA accumulated to a higher level than BP. While the abdominal muscle showed greater accumulation of BP at the molt, the digestive tract, hepatopancreas, and abdominal muscle showed greater uptake of BA in newly molted animals.

When comparing the rate of discharge of aromatic hydrocarbons in marine invertebrates (Harris et al., 1977), copepods appeared to lose naphthalene rapidly in the first 48 hr of depuration. Brown shrimp eliminated a large amount of naphthalene in minutes (Anderson et al., 1974), whereas Neff and coworkers (1976) demonstrated that initially brown shrimp rapidly released naphthalene followed by a long period of gradual depuration. In other studies, the blue crab slowly depurated aromatic hydrocarbons (Lee et al., 1976); in two days, the blue crab lost about half of the benzopyrene accumulated over the previous two days. Benzopyrene loss in grass shrimp was slower than in blue crab and may be equated with the release from the clam, Rangia cuneata (Neff and Anderson, 1975), which lost 10% of its PAH burden in 6 days. Lee and others (1972b) found that fish discharged half of their benzopyrene burden from gut and liver in the first day of depuration. The blue crab released about half of its burden from hepatopancreas but showed little change in stomach benzopyrene after two days of depuration (Lee et al., 1976).

Our investigation as well as previous studies using invertebrates and fish suggests that aromatic hydrocarbons are rapidly accumulated in marine animals and that their release is dependent upon the number of benzenoid rings present. Naphthalenes are rapidly discharged, while PAHs are slowly released. Benzathracene appears to depurate more easily than its related carcinogen, benzopyrene. Further investigations of several aromatic hydrocarbons with differing numbers of benzenoid rings in a variety of marine animals would help in clarifying PAH contamination of the marine environment. Contradictions observed in the limited study of these compounds in marine animals can only be eliminated by further studies.

ACKNOWLEDGEMENTS

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AN ECOLOGICAL PERSPECTIVE ON HUMAN FOOD WEBS

by

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ABSTRACT

Summaries in this text illustrate the complexity of human food webs and indicate the present lack of understanding in the area of human food web ecology. Increases in the numbers and rates of introduction of hazardous chemicals in effect may reduce the ability of organisms essential to food webs to react in a timely manner. Many facets of this complex problem are being addressed at present, but no structured program exists.

It is recommended that an ad hoc committee be sponsored by the U.S. Environmental Protection Agency (EPA) to organize ecological research needs and priorities on the subject of xenobiotics in human food webs. Also, a strategy should be developed to assist in the implementation of an information management system.

INTRODUCTION

Chemical constituents of food relevant to food safety include a wide spectrum of substances that are introduced through varied routes. The purpose of this document is to describe the need for an understanding of the origin of environmental contaminants in the human diet. The complex subject might best be approached through a basic knowledge of the movement of xenobiotics through food chains to man.

Food-chain studies can provide a framework for an integrated approach to ongoing research. Results from such studies could be applied to develop a strategy to prevent the exposure of man to xenobiotics through the food chain.

The development of such a strategy requires a comprehension of the transport and fate of food contaminants that move through the abiotic (physical) compartments and biotic (living) compartments of the food chain. Of particular complexity are the interfaces of physical compartments, i.e., air and water, or water and soil.

The biotic compartments of food webs are poorly understood. Processes, such as biotransformation of chemicals and the structure of food webs, are not well-known. Very little is known about diet-related human behavior, such as actual dietary intake, nutritional characteristics and regional preferences, and changes in dietary habits. Thus, the prediction of human exposures becomes a speculative art. Because human dietary intake is poorly elucidated, there is an unclear relationship between human nutrition and ecological food-web trophic-dynamics.

Food chains that directly or indirectly involve human consumers are influenced by many factors. Some of the penultimate problems are resolvable, but others present interactions which are beyond resolution with current techniques and have little more than an empirical basis for investigation. These questions include broad areas of human nutrition, induction of pathological process by toxicants, and oncogenesis.

The suggested methods of approach include the monitoring of the pathways of xenobiotics in "human food chains" by using the trophic-dynamic/systems theoretic as the basis for the program of research on food webs. This theory incorporates and integrates humans into the energy flow in the ecosphere rather than isolating them.

The impact of human activities since the industrial revolution has affected global climates, atmospheric and oceanic nutrient cycling, crop nutrient cycling, and perhaps even the energetics that drive these cycles. Natural resource exploitation and the attendant problems associated with the rapid population increase and elevated living standards in developed countries have greatly accentuated the introduction of hazardous levels of xenobiotics into humans. The rates of introduction of compounds and the enormous scale of industrial activity has exacerbated the "ecological" problems and greatly reduced the time available to react to these insults. The environmental legislation which has been enacted in recent years is a response to the threat posed by gross chemical introductions.

The basis for a food-chain research program presently exists. A number of individual projects are already underway in the EPA Office of Research and Development. The organization of these ongoing projects may not be strictly directed toward food chains. However, with careful coordination and planning by scientists, the effort may result in significant accomplishments in relating and applying ecological theory to problems regarding human diets.

Background

Pollutants can reach man in a variety of ways--in the air he breathes, the food and water he ingests, through his skin, from his surroundings (including air, water, and solids), and from a combination of routes. There can be continuous exposure, intermittent but repeated exposure, or sporadic exposure; concentrations may remain constant or vary greatly;

exposures may result from direct, intentional, or unavoidable applications (i.e., food, drugs, water); and exposures may be effected through complex environmental pathways.

A thorough understanding of the pathways of a contaminant in the environment affords a reasonable basis for estimating human exposure. Understanding of what happens to a chemical in the environment provides the only rational basis to prevent or minimize human exposure through food chains. Prudence dictates that it is much more desirable to limit the level of exposure to prevent ill effects than to attempt to reduce exposure to an acceptable level of chemicals after an effect has occured. For example, if certain physical conditions enhance the growth of toxin-producing fungi, perhaps such conditions can be avoided. It is known that cotton grown in irrigated desert areas often habors extensive growths of *Aspergillus flavus*, which produces aflatoxin in the cotton seed. (Cotton seed meal is a component of human food and animal feeds.) Another example is the fallout of toxic elements (metals) in flyash from power plants and from emissions of smelters of refineries, which may result in crop uptake of excessively high levels of undersirable trace substances. Attention to power plant placement or control of crops planted in the vicinity of such facilities could eliminate or reduce human exposure to such material. The knowledge of the transport and alteration of a chemical can be used to avoid or prevent exposure, i.e., the sequestration of mercury by sediments and improved methods and processes in handling and using such organics as pentachlorophenol.

Chemicals may be transported and transformed in complex ways (Figure 1). Once released (volatilized) into the environment, they may be transported in currents of air or water, or in association with solid particles. They may be transformed into hazardous compounds by chemical or biochemical reactions, diluted by diffusion, or concentrated by physical or biological processes. Biochemical transformation in the cell/organ system may produce oncogens from moderately toxic precursors. Human exposure to chemicals takes place not only at point of use or discharge of the chemical but also at points distant in the space and time horizon.

To assess the total magnitude of human exposure, we must trace the movement as well as the transformation of a chemical from the point of its release in the environment to the site where it may be degraded as a less harmful substance.

Figure 2 shows pathways by which chemicals reach man. In this simple scheme, the environment is represented by "boxes" or compartments through which chemicals move at various rates. Arrows between the boxes denote transfer between compartments. To estimate the concentration of a chemical in a compartment, we must know not only the rate of release of the chemical (a function of its use patterns), but also its retention time within the compartment. The retention time is determined by the rates of transfer to

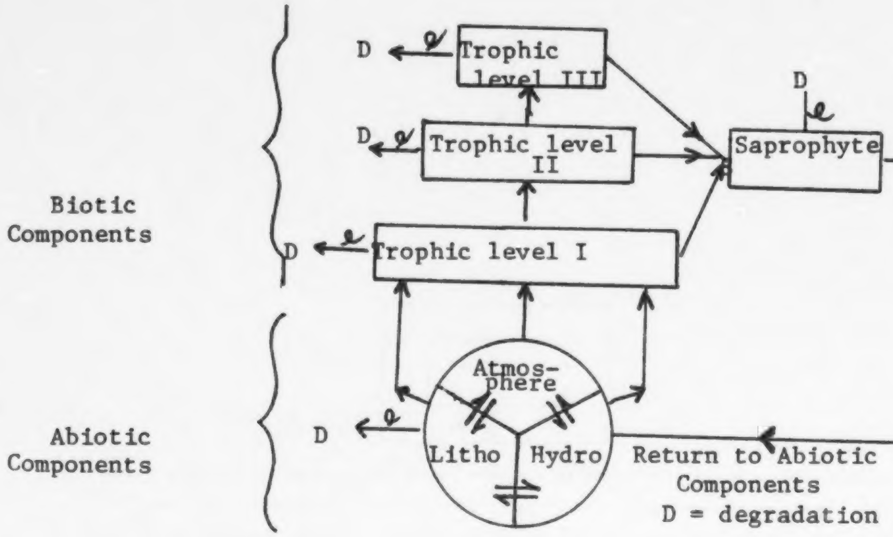
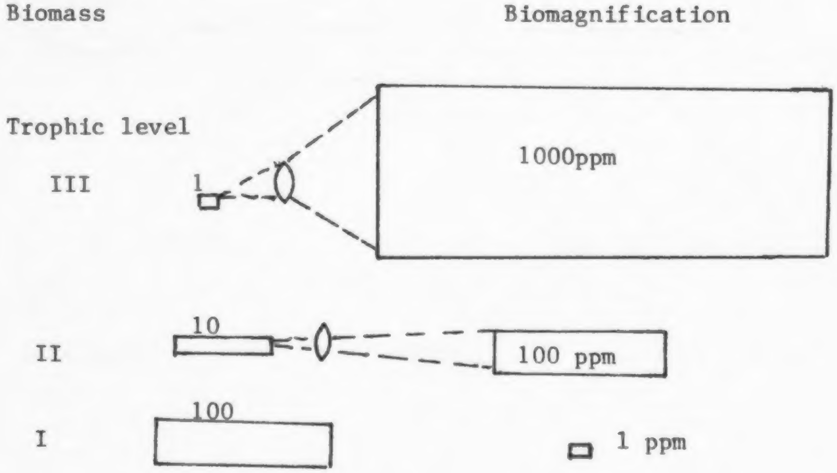

D
Trophic level III
D
Saprophyte
D
Trophic level II
Biotic Components
D
Trophic level I
Atmosphere
Abiotic Components
D
Litho
Hydro
Return to Abiotic Components
D = degradation

Figure 1. Model of chemical transport and degradation in biosphere. (from Robinson, 1973).


Biomass
Biomagnification
Trophic level
III
1
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II
10
100 ppm
I
100
1 ppm

Figure 3. Biomagnification/bioconcentration in a food web (from De Santo, 1978).

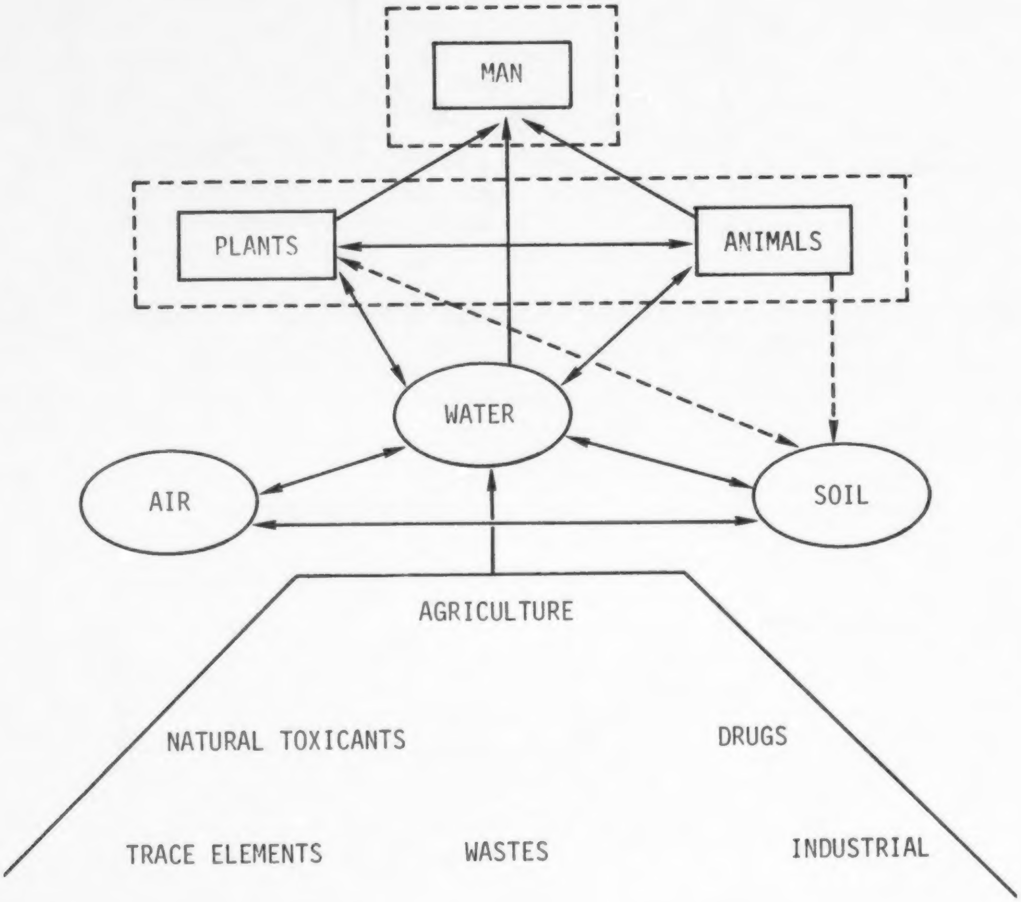
MAN
PLANTS
ANIMALS
WATER
AIR
SOIL
AGRICULTURE
NATURAL TOXICANTS
DRUGS
TRACE ELEMENTS
WASTES
INDUSTRIAL

Figure 2. General diagram of chemical flow in food web.

to and from other compartments, by the rates of alteration of the chemical, and by diffusion processes within the compartment. Thus, to understand the behavior of chemicals in food chains, we should study the processes of dispersion, the transfer between and within compartments, the chemical and biological transformations in compartments, and bioaccumulation in the biota of a compartment.

Many environmental processes result in dispersion, dilution, or degradation of chemicals. A most important reconcentration process in human food webs is "bioconcentration," in which plants and animals may accumulate certain chemicals to levels much higher than those in their ambient environment (Figure 3). Other processes include the absorption of chemicals onto airborne or waterborne particles and the concentration of certain chemicals from liquid effluents into sewage sludge and subsequent possible entry into human food crops.

Transport of chemicals takes place on various geographic scales. On both regional and global scales, an important transport takes place in the atmosphere. For some substances with low reactivity, passive transport and diffusion are the factors of importance. The processes that remove chemicals from the atmosphere are less well-known. Some substances diffuse upward and are degraded by ultraviolet radiation. Others diffuse downward to be adsorbed onto the surfaces of suspended particles; others are dissolved in water droplets and returned to earth in rainfall. In some cases, global atmospheric processes may include cycles of elimination and reintroduction of a substance, as the balance and flow are determined by the air and water or air and terrestrial interface reactions. In any case, man is the ultimate food-chain receptor of such chemicals (Figure 1).

Thus, as in the atmosphere, the transport and retention time of chemicals in water may be controlled by processes which take place at phase boundaries and which, as yet, are poorly understood. On balance, the direction of movement is toward the oceans, although processes, such as chemical precipitation or adsorption on solids, may result in deposition in "sinks" that intercept the movement entirely or "reservoirs" that delay the movement. These sinks and reservoirs contribute to accumulations in the local biota, in food organisms, and ultimately in humans.

Pollutants can be actively transported in biological systems. On a regional basis, migrating birds or fish may carry small amounts of pollutants for long distances. The amounts involved are for the most part insignificant in terms of global or regional redistribution. On the other hand, the quantities of pollutants carried in or on plants or animals used as food may be of major concern in relation to human exposure.

In contrast to the atmosphere or water, the soil compartment serves both as a reservoir which receives and disperses pollutants and as a "chemical reactor" in which transformations take place (Figure 4).

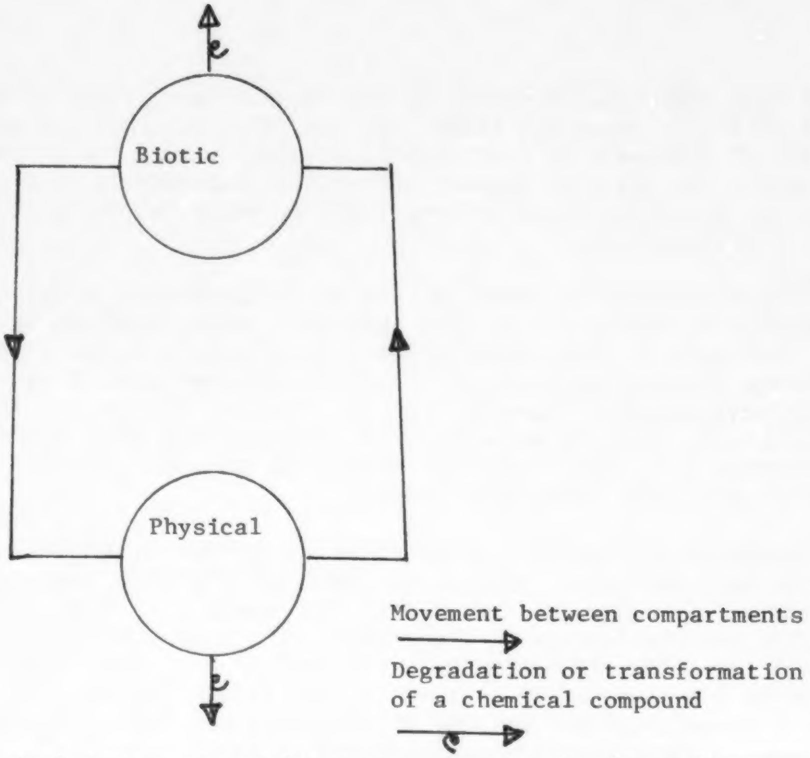

Figure 4. Chemical transport model between compartments (from Robinson, 1973).

Substantial quantities of chemicals released into the environment reach the soil either through direct application or transfer from air or water. It should be recognized that there is a constant interchange between the compartments of the environment. Consequently, a chemical applied to soil may transfer in a reversible process to air or to water through run-off and leaching. Nonetheless, the soil becomes an important reservoir for many chemicals (Figure 2).

Three major interactions or processes are of concern in soil: sorption, leaching-diffusion, and alterations through chemical and biochemical processes. Obviously these interactions affect the plants and animals consumed as food. For example, it is reasonable to ask how rapid and in what quantity a chemical may move from a landfill disposal site as a result of precipitation percolating through the soil profile or of ground water moving through the landfill. If leached in considerable quantities, the chemicals may subsequently reach aquifers used as human water sources or be carried into streams and bodies of water containing food-chain biota. Adsorption plays a very important role in leaching and diffusion behavior so that strongly adsorbed compounds are found to be poorly leached by water. Substances, such as higher molecular weight halogenated organics, i.e., polychlorinated biphenyls and DDT may show little movement even after several years of exposure to percolating water.

Many organic chemicals become solids at ambient temperatures. The kinetic motion of the molecules causes them to have a finite vapor pressure even at these temperatures. As temperatures increase, the transition from solid to vapor with or without an intermediate liquid phase tends to result in increased vapor pressure. The vapor pressure then is related in a complex fashion to the rate of evaporation of the compound and to its tendency to exchange across air/water or soil/air interfaces or pass directly into the atmosphere. The chemical in the vapor state will quickly establish an equilibrium state of adsorption on particles suspended in the atmosphere. Photodecomposition may occur in either the sorbed or vapor state. The contributions to food chains are indirect but nevertheless significant via the evaporation-volatilization of hazardous substances.

The human habitat plays a role in the transport of polluting chemicals. The structure of the outdoor environment of towns and cities provides semienclosed spaces from which chemicals diffuse slowly. Paved areas, drainage networks, and sewage systems constitute a pathway for transporting chemicals out of urban areas. Sewage treatment plants separate the dissolved and solid-phase components of the chemical mixture. Disposal of the dissolved materials into waterways and discharge of the sewage sludge on land may lead indirectly to significant human exposures via food chains. These urban subsystems may be ultimately more important in determining human exposure to chemicals than the rural environment.

The physical behavior of these substances in the environment must be understood, as well as their fate and accumulation. Three major processes are involved in breakdown and metabolism: photochemical, chemical, and biologically mediated alterations. Photochemical breakdown induced by absorption of light will induce alterations. For some substances, the quantum efficiency is high and consequent breakdown is rapid. Chemical breakdown, on the other hand, is dependent upon molecular structure. If a chemical is susceptible to nucleophilic attack, oxidation, or hydroxylation, alterations can occur fairly rapidly. Similarly, a chemical may undergo hydrolysis, the rate of which will be dependent on pH, temperature, and the presence of catalytic sites. For example, certain organophosphates in soil are rapidly hydrolyzed following sorption (presumably the clay affords catalysis for this reaction). On the other hand, halogenated hydrocarbons tend to be more refractory toward purely chemical reactions and hence persist for long periods in food-chain compartments.

Alterations from biologicaly mediated reactions are variable. Although considerable research has been devoted to metabolic studies and investigations of alterations occurring in the environment, complete understanding for all but a few compounds is lacking. In describing the behavior of organic compounds in the environment, observers have reported that compounds partition into a particular phase. The partition coefficient of an organic substance from water into a lipid solvent may be related (in certain instances) to its biological activity. It has been observed that the partition coefficient in octanol/water correlated very closely with the propensity of an organic substance to accumualte in fatty tissues of organisms. It has been demonstrated that the octanol/water partition coefficient is related to the adsorbability of a compound by soil organic matter and certain clays. This can be of predictive value in studying exposure of human food to hazardous chemicals.

An important example of partitioning is the phenomenon of bioconcentration. Many aquatic plants and animals are able to concentrate persistent chemicals, heavy metals and lipophilic organic compounds into their tissues to levels many times those in the ambient water (Figure 2). In extreme cases, such as the concentration of cadmium by shellfish, and DDE and PCBs by fish, the concentration factors may be as high as one hundred thousand or even one million times the ambient levels. For such chemicals, consumption of contaminated fish or shellfish is often the principal route of human exposure. Although many measurements of bioconcentration factors have been made, there is still an incomplete understanding of the biological mechanisms that determine the exact degree of concentration achieved for various chemicals. However, it is now possible to estimate if some chemicals will bioconcentrate based upon structure activity relationships. Structure activity correlations can be used to predict biodegredability under waste-treatment and environmental conditions and to provide valuable insight into the assimilating capacity of the environment.

Techniques are well-established to predict the toxicity of organic chemicals on structural properties such as lipid solubility and electronegativity and an appropriate structure-activity correlation. With respect to toxic effects to aquatic organisms, the major research need is an adequate data base of toxicological information from which necessary quantitative correlations can be established. The establishment of this predictive capability will permit estimates of toxicity. Detailed toxicological studies can be made on those chemicals with the greatest potential for adverse reactions, including contaminants of the human food-chain.

The problems associated with acid rain are related to food web dynamics. This is a serious concern because of the increased heavy metal (Pb, Cd, As, Se) uptake by food plants with altered soil characteristics. Also, the changed pH of atmospheric moisture results in increased solubility and consequently increased foliar absorption of heavy metals by leafy vegetables and animal forages. This is a relevant example of a disturbance in an ecological compartment felt throughout the bioshpere with man as the witless target species.

Human exposure estimation is an area of principal concern in attempting to resolve some of the food-chain dynamics. Human nutrition data should be collected on a national as well as a regional basis. Significant regional differences in food intake are apparent. When this information becomes available and the quantities of ingested foods are known, food-chain pathways of pollutants can be ascertained. In order to assess the dietary intake of chemicals present in food, the amount of a given substance present in different foods can be measured. In addition, it should be determined if the source of pollutants is environmental or industrial (food processing). By combining this information with data on food consumption, estimates can be made. Factors to be taken into account in this approach include: variations in dietary patterns, quantities of given foods consumed by different age or ethnic groups, and variations in concentrations of a given chemical in different foods at different times.

A substance that occurs in more than one compartment of the human food chain (Figure 2) may promote exposure through several routes varying in form and quantity; hence, total exposure can only be estimated by combining the separate sources and measuring them in different trophic levels.

Food-Chain Related Programs

The problems associated with the addition of chemicals are not easily resolved because of the complexity of the systems in terms of the numbers of interacting organisms and "level of organization" through which these compounds and their metabolites pass. In related research, we must also consider how and where these compounds bioaccumulate, whether or not the various organisms are capable of depurating, and if the compounds are altered or transformed to produce carcinogens, mutagens, and teratogens.

The techniques for recognition of carcinogens in human food organisms and those organisms indirectly important in food webs are under development by EPA researchers. At present, these techniques and their causal association are being studied by a few investigators. This research has been pursued by scientists at EPA, the Food and Drug Administration (FDA), the National Cancer Institute (NCI), and the Smithsonian Institution. The induction of carcinogenic processes and the description of inducing mechanisms in food web organisms are important areas of investigation.

There is a limited program of biological tissue monitoring by EPA scientists (mussel watch) for toxic substances. U.S. coastal areas range from the heavily contaminated to almost pristine and are monitored for data on the incidence of neoplasms and tissue degeneration in four species of bivalve mollusks. This effort appears to be the only investigative program of its type which deals with anomalies in organisms related to the human food webs. However, low level funding has hampered its effectiveness. Other EPA research projects underway in 1978 are listed below.

Atmospheric Research

Airborne contaminants from mobile sources in human food crops (plants), animals forages and meat animals, dairy products (these include heavy metals, hydrocarbons, oxides of nitrogen and sulfur, ozone, and trace metals from emission controls).

Atmospheric source influence on ground water quality from NOx, SO_2, O_3, HCN, CO, trace metals: Mn, Ni, Ru, Ir, SO_2; heavy metals: Pb, As CN, Se, Cd.

Effects of atmospheric contaminants on marine food organisms.

Bioaccumulation and concentration, sediment uptake and release, levels of contaminants in primary producers and consumers.

Effects of multiroute exposure (ingestion) to man of Cd and Pb.

Atmospheric sources to human food chain from petrochemical complexes; incidence of tumorogenic disorders in populations of food-chain organisms.

Dendrochronology - relation of time of depositon to pollutants.

Exposure assessment of pollutants in oil and water exposure compartments; predictive model of volume and distribution in environmental compartments.

Response of critical receptor organisms (including man) from atmospheric toxics.

Atmospheric chemical fluxes relative to fluxes in sediments.

Surveys of atmospheric hazard substances in the Great Lakes.

Fluxes of hazardous substances in Saginaw Bay from atmosphere.

Toxics uptake by phytoplankton in the Great Lakes.

Terrestrial ecosystem pathways and nutrient cycling effects from airborne contaminants (pesticides, trace elements, metals, and gaseous air pollutants).

Pollutant pathways in plants, soils, and animals.

Energy-Related

Determination of toxicity and bioaccumulation of polyaromatic hydrocarbons from energy sources in aquatic and terrestrial animals.

Effect of shale oil development by-products on aquatic organisms in the food chain.

Effects of pollutants from coal gasification and liquefaction on aquatic and terrestrial life (including man) through the food chain.

Impacts of biocides on food webs through the processes of bioaccumulation and interaction with sediments.

Effects of compounds on histopathology of marine organisms (fish), and teratology of organisms from halocarbon exposure.

Effects of carcinogens from shale oil in the marine food web.

Effects of SO_2 and other pollutants on grasslands.

Sewage wastes effects and toxic uptake by crop plants.

Fate and transport of energy-related pollutants in biological systems.

Characterization of ground water geohydrology in relation to oil-shale processing.

Food Crop Pathways

Models to predict behavior of agricultural chemicals and animals wastes in major non-irrigated crop regions under various edaphic and climatic conditions, i.e., bioaccumulation and bioconcentration of chemicals in food organisms.

Trace element transport and organic biotransformation resulting from passage through plant and soil systems.

Coal-fired power plant vicinity sampling and analysis of soils and plants for Hg, Pb, Cd, and As.

Drinking Water Contamination

Effects of trihalomethanes/halorganics (as carcinogens, mutagens, teratogens) in drinking water.

Alternate disinfection chemical by-products as carcinogens, mutagens, teratogens.

Water/food supply availability of inorganics and metals: Hg, As, Ba, Se, Cd and their influence on cardiovacular disease.

Effects of asbestiform fibers in drinking water.

Effects of geochemicals and industrial organics from water supply.

Persistence and availability of pesticides (from soil to food chain organisms).

Storage, depuration, and excretion of halogenated and nonhalogenated pesticides in animals and man.

Aquatic Ecological Effects

Bioaccumulation of organics in primary producers and primary consumers.

Mass balance for toxics in the Great Lakes ecosystem; bioaccumulation of hazardous substances in fish.

Hazardous substances and heavy metals uptake and release in sediments and in benthic algal communities (marine and freshwater).

Analysis and tracking of organic chemicals (Mirex, Kepone, PCPs, and PCBs) in fish of the Great Lakes.

Accumulation of DDT and PCBs in food fish.

Pathway of asbestos to human water sources.

Land bioaccumualtion and alteration of communities from wastewater and sludge application.

Distribution and source of PCBs in dredge spoils.

Sediment processes, fate, bioaccumulation, and toxicity of Kepone in food-chain organisms and sediment processes in the James River estuary.

Fate and effects of petroleum hydrocarbons, transuranics, pesticides and heavy metals in organisms in the estuary.

Structure activity studies from which to make predictions on bioaccumulation and toxicity of certain classes or organics.

Water Quality

Human health effects associated with treatment and disposal of wastewater with reference to persistent organics (and particularly PCBs) in food web; Cd, Pb, and trace metals translocated from sludge to soil, to plants, to man; persistent organics in human food web organisms from wastewater sources.

Development of and testing for persistence of hazardous substances in water.

Bioaccumulation and health hazard test protocols for pollutants.

Literature search for documentation of water quality for shellfish using chemical and biological data.

Ecological processes and effects of land application of municipal wastewater and sludge and non-point runoff on plant and animal communities including bioaccumulation, population dynamics, etc., in aquatic systems.

Land application sewage sludge and pathways of contaminant through soils, groundwater, surface water, plants, and animals to humans as evidenced by food chains.

Food-chain effects of heavy metals on public health (epidemiology).

Monitoring

Development of monitoring methods for toxic industrial and municipal wastes and hazard evaluation applicable to human populations and aquatic food-chain organisms.

Fate and effects of organics in aquatic systems, particularly carcinogens in estuarine and marine systems.

Routes and rates of pesticide movement through ecosystems to man.

Bioaccumulation and concentration of chemicals in estuarine food web organisms.

Development and use of aquatic indicator species of carcinogens, mutagens, and teratogens in food web.

Transport of substitute chemicals (pesticides) and degradation products in model systems.

Hazardous chemicals and pesticides bioaccumulated in terrestrial, estuarine/marine, and freshwater systems and organisms.

Substitute chemical (pesticides) interaction with other chemical agents, pathogens, environmental conditions relative to disposition in microcosms (rates and pathways of accumulation).

Other Federal Programs

There are other Federal Programs outside EPA that are relevant to food-chain research. The Food and Drug Administration has research projects related to the sources of various contaminants which directly enter human food webs. These projects include the following:

PCBs in food and food packaging materials
Organochlorine pesticide residue analysis in fish
Herbicides and fungicides in the human diet
Total diet identification of pesticide residues
Dioxins as food contaminants
Mercury, cadmium, lead, and heavy metal contaminant in food
Chlorinated dibenzofurans as food contaminants
Sewage-derived chemicals
Food Contaminants
Pesticides and metals in food

Radionuclides in food
Heavy metals in processed foods
Total diets in adults, infants, and toddlers
Carbamates in foods
Plant toxins, biogenic toxicants, and marine toxicants in foods.

Atmospheric-Food Chain Inputs--There have been assessments of the problems associated with a portion of this general area. The National Academy of Science (NAS) publication, *The Tropospheric Transport of Pollutants and Other Substances to the Oceans* (1978), outlines the problems. A brief synopsis follows:

I. The current data are insufficient to design a comprehensive and sound monitoring program; therefore, research lines must be followed until some limited set of quality data make the definition of monitoring network feasible.

II. There is a great need for atmospheric and oceanic concentration and composition data on such pollutant groups as:

(1) *Metals*, in particular, their concentration in surface slicks and water layers, and a description of their continental sources.

(2) *Halogenated hydrocarbons*, in particular, the high molecular weight hydrocarbons, such as the chlorinated hydrocarbons. It is important to measure concentration in phytoplankton and zooplankton to determine biomagnification of these compounds.

(3) *Low molecular weight halocarbons and monohalomethanes*. Their source is probably oceanic. These can be divided into two groups: short and long residence time.
Short residence time - halocarbons (6 months to 1 year). Their source is probably from rivers and not the atmosphere.
Long residence time - halocarbons, such as the low molecular weight CCl_4, $CHCL_3$, $(CH_3)\ CCL_3$, would remain in solution when dumped into rivers or oceans. Oceans act as a reservoir for these compounds from atmospheric sources, for example.

(4) High molecular weight halocarbons. Removal by attachment to aerosol particles may be important for high molecular weight chlorinated hydrocarbons. There is evidence that aerial fallout on a 50 x 200 km nearshore area in the Southern California Bight for DDT and PCBs exceeds runoff and wastewater (sewage outfalls) inputs to a comparable area.

CONCLUSIONS

EPA Environmental Research Laboratories have a unique capability to advance the food-chain research. Their research objectives might emphasize and elucidate the following areas:

(1) Environmental samples and tissues from human populations and establish banks for monitoring.

(2) Establishing a bank for the tissues of regional terrestrial and aquatic species to monitor the population fluctuations.

(3) The fate and transport of xenobiotics through compartments and bioaccumulation and a transformation of xenobiotics by the biota.

(4) The global atmospheric processes of elimination and reintroduction of a substance with the balance and flow determined by the air and water or air and terrestrial interface reactions.

(5) A working model of the urban food web--the cycling and pathways of industrial contamination of biospheric compartments.

(6) Data on food consumption and amount of chemicals present in food to establish human exposure estimates.

(7) A comprehensive model and a scientific information access system relative to food chain xenobiotics.

(8) An environmental forecasting and technology assessment of human food-chain problems.

EPA laboratories should be given adequate funding and personnel to meet these objectives. In such a comprehensive program, the laboratory research scientist should be able to set priorities and determine the validity and nature of such projects.

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THE CELLULAR FATE OF BENZO(A)PYRENE

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ABSTRACT

Recent work in mammalian systems emphasizes covalent binding of chemical carcinogens to DNA as the initiating event in the process of cell transformation. In our paper cellular and molecular aspects of carcinogen action are summarized particularly with respect to covalent binding of derivatives of benzo(a)pyrene (BaP) to DNA and RNA in hamster embryo cell cultures. After addition of BaP, the binding to RNA proceeds rapidly and follows a linear time course for at least 48 hr, presumably because of a lack of an RNA repair mechanism. In contrast, after approximately 18 hr of incubation with benzo(a)pyrene, the extent of binding to DNA reaches a plateau, reflecting equilibrium between *de novo* binding and DNA repair processes. A detailed analysis provides evidence that both stereoisomers of benzo(a)pyrene 7,8-dihydrodiol 9,10-oxide (BaPDE) are involved in covalent binding to hamster embryo cellular nucleic acids.

The relevance of these studies in mammalian systems to the marine environment and marine organisms is discussed. The possible consequences of bioaccumulation of polycyclic aromatic hydrocarbons in marine organisms and the metabolic processes and neoplasia induction in these organisms are explored in terms of potential hazards to humans.

INTRODUCTION

During this meeting, it has become apparent that oceans are the recipients of innumerable foreign organic compounds that occur as environmental pollutants. Consequently, it is not surprising that this contamination affects marine biota. Deposition of these pollutants in tissues, bioaccumulation, metabolism, degradation or depuration, detoxification, and neoplasia induction are well-documented in marine organisms. Of additional concern is the possible impact of this type of marine pollution on human cancer induction. In the complex marine food web, contaminated organisms may be a potential food source for other organisms at higher trophic levels and thus eventually appear in human food sources. These considerations have major implications in terms of both marine ecology and human health.

Although many studies have focused on the mixed function oxidase (MFO) enzyme system in marine organisms, there has been considerably less attention to the cellular and molecular aspects of chemical carcinogenesis in marine species. Historically, basic discoveries in this area have primarily involved nonmarine organisms. In the present paper, an attempt is made to evaluate the status of our understanding of the cellular fate of certain carcinogens in mammalian systems. Emphasis will be placed on the compound BaP and on studies analyzing its covalent binding to mammalian cell DNA.

Celluar Events in Mammalian Systems

Figure 1 summarizes the cellular fate of BaP or related carcinogens in mammalian systems. The first critical step in the encounter between a cell and a potential carcinogen is metabolism of the compound. This subject has been discussed in detail, with respect to fish, during this meeting. Most of the metabolites that are formed are detoxification products. During this process, however, highly reactive activated intermediates are formed which, unless further metabolized, can act as "proximate" and "ultimate" carcinogens. Within this context, carcinogenesis can be thought of as an error in drug detoxification. Ultimate carcinogens are highly reactive electrophiles (Miller, 1970) that can bind covalently to nucleophilic residues in cellular macromolecules--DNA, RNA and proteins (Brookes and Lowley, 1964). At present, it is not known with certainty which target is critical in terms of the process of cell transformation. Current studies favor DNA as the critical target but its role in transformation is not understood. A number of laboratories have shown that when DNA is modified with chemical carcinogens, there is impairment of not only DNA replication but also RNA transcription (Weinstein, 1976). It is possible, therefore, that serious distortions in gene transcription and the control of gene expression occur as a result of the modification of cellular DNA.

How do organisms protect themselves against this attack of foreign materials on DNA and thereby avoid genetic damage? Nature has evolved a highly efficient and complex enzyme mechanism, called DNA repair, which can recognize damaged regions of DNA, excise and then repair them (Friedberg *et al*., 1977). If DNA repair systems operate efficiently and with high fidelity (error free repair), the host has solved the problem. If, in contrast, the repair is incomplete, or "error-prone," then mistakes are likely to occur in the daughter strand when the DNA replicates, and mutations will occur in the daughter cell. The damage to DNA by carcinogens and its consequent repair provide a basis for some of the short-term tests for carcinogens that assay for mutagenicity or induction of DNA repair.

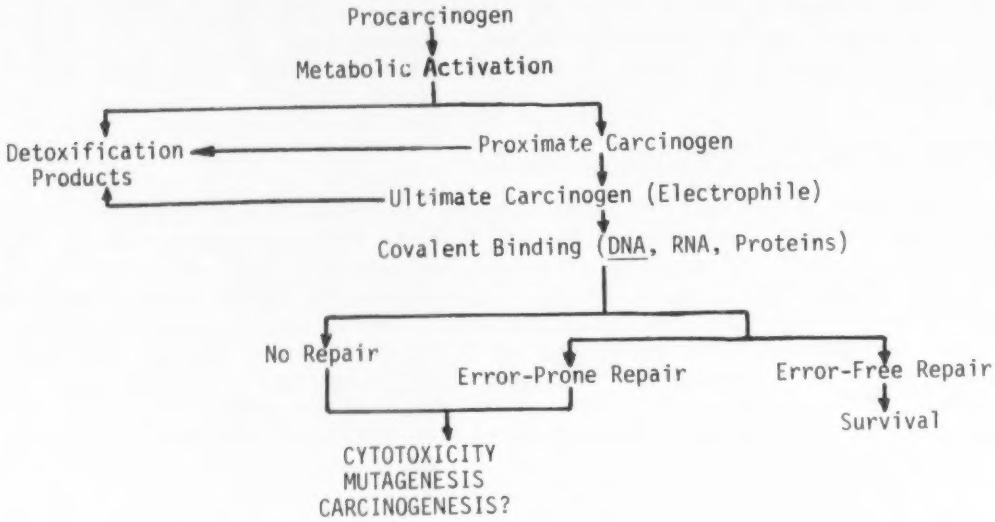

Procarcinogen
Metabolic Activation
Detoxification Products
Proximate Carcinogen
Ultimate Carcinogen (Electrophile)
Covalent Binding (DNA, RNA, Proteins)
No Repair
Error-Prone Repair
Error-Free Repair
Survival
CYTOTOXICITY
MUTAGENESIS
CARCINOGENESIS?

Figure 1. Cellular fate of carcinogens in mammalian systems.

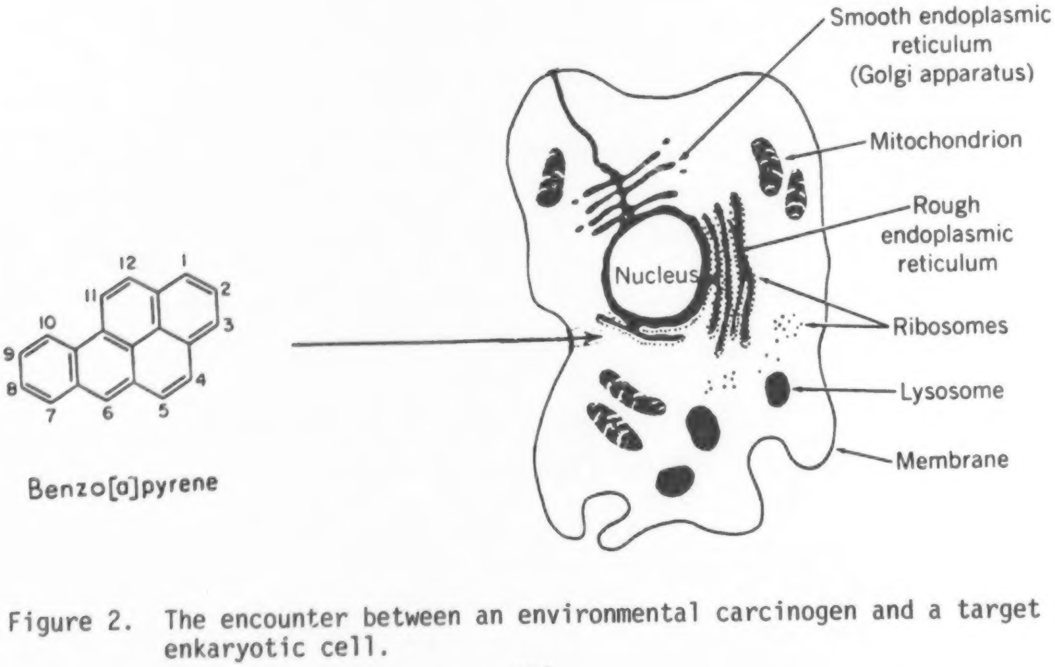

12
1
2
3
4
5
6
7
8
9
10
11
Benzo[a]pyrene
Smooth endoplasmic reticulum (Golgi apparatus)
Mitochondrion
Rough endoplasmic reticulum
Nucleus
Ribosomes
Lysosome
Membrane

Figure 2. The encounter between an environmental carcinogen and a target enkaryotic cell.

Our laboratory has focused on the molecular details of the covalent binding of chemical carcinogens to DNA that appear to reflect the initating events in cell transformation. The study of these covalent adducts rather than simple metabolites has several advantages since cellular DNA acts as a trapping agent for ultimate carcinogenic metabolite(s), and the extent of binding may provide an index of the potential potency of the compound in question.

Benzo(a)pyrene in Rodent Cell Cultures

The most extensively studied polycyclic aromatic hydrocarbon (PAH) carcinogen is BaP. As presented in Figure 2, this carcinogen enters the cell most probably by passive diffusion through the lipid layer of the membrane (Burnette and Katz, 1975). It reaches the endoplasmic reticulum and nucleus and induces the so-called aryl hydrocarbon hydroxylase (AHH) or MFO enzyme system. This monooxygenase cytochrome P-450 system oxidizes BaP at a variety of positions to form more than 35 metabolites (Yang and Gelboin, 1977a) (Figure 3). Most of metabolites are detoxification products, while some bind covalently to DNA, RNA, and proteins.

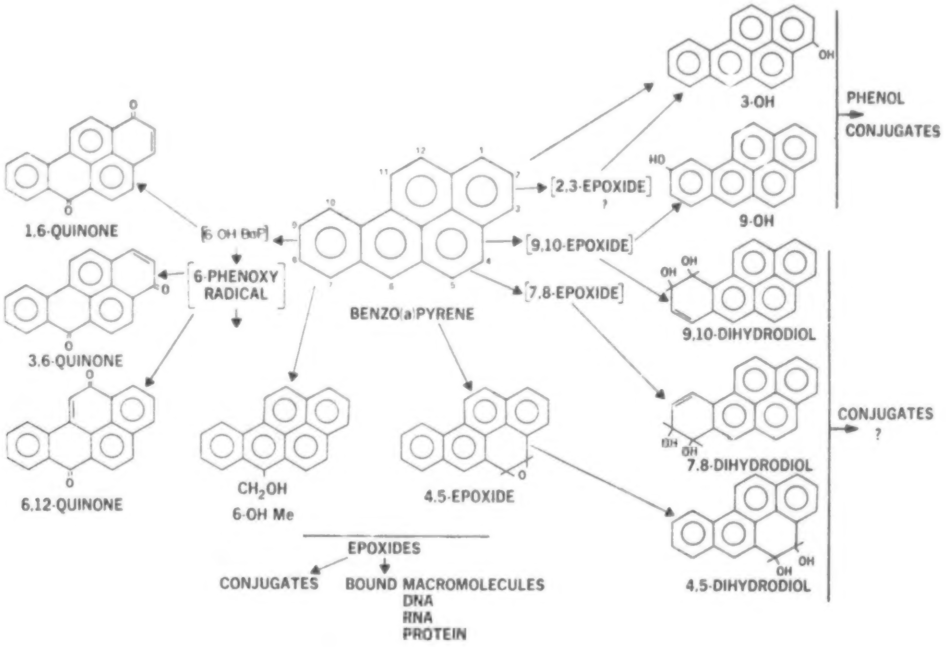

1,6-QUINONE
3,6-QUINONE
6,12-QUINONE
[6 OH BaP]
6-PHENOXY RADICAL
BENZO(a)PYRENE
CH$_2$OH
6-OH Me
4,5-EPOXIDE
EPOXIDES
CONJUGATES
BOUND MACROMOLECULES
DNA
RNA
PROTEIN
[2,3-EPOXIDE] ?
[9,10-EPOXIDE]
[7,8-EPOXIDE]
3-OH
9-OH
PHENOL
CONJUGATES
9,10-DIHYDRODIOL
7,8-DIHYDRODIOL
4,5-DIHYDRODIOL
CONJUGATES ?

Figure 3. Benzo(a)pyrene metabolism (Selkirk et al., 1974).

In order to study this covalent binding, we exposed various confluent rodent cell cultures to 1 μg/mℓ of (^{14}C)-labeled BaP. After 24-hr incubation, cells were harvested and nucleic acids extracted and rigorously purified to exclude contaminants and noncovalently bound material. The amount of carcinogen covalently bound was determined by the radioactivity of the purified nucleic acids (Table 1). Table 1 indicates that the K-22 epithelial rat liver cells bind BaP to DNA at a rather low level, i.e., one residue of BaP per 150,000 nucleotides. We have found considerable variations between cell types. Primary hamster embryo cells were chosen for more detailed studies of BaP binding, since the modification was considerably higher (1/45,000), and hamster embryo cells are readily transformed in culture by BaP (Berwald and Iachs, 1965; DiPaolo and Donovan, 1967). These studies have been published (Grover _et al_., 1974) and results are summarized below.

TABLE 1. THE EXTENT OF _IN VIVO_ BaP BINDING TO CELLULAR NUCLEIC ACIDS

Cell Culture	Nucleic Acid	Extent
K-22 epithelial rat liver cells	DNA+RNA	1/150,000
Hamster embryo cells (confluent primary culture)	RNA	1/30,000
	DNA	1/45,000

The time courses of covalent binding of BaP to RNA and DNA in hamster embryo cell cultures are compared in Figure 4. The binding to RNA proceeded rapidly and followed an approximately linear time course for at least 48 hr. This indicates that metabolites are available for covalent binding during this period. In contrast, after approximately 18 hr incubation with BaP, DNA binding reached a plateau. This plateau is due to an equilibrium between _de novo_ binding and DNA repair processes.

These studies with radioactively labeled carcinogen-bound nucleic acids, of course, do not provide information on the nature of the intermediates and nucleoside adducts formed. In order to understand the relation between the formation of covalently bound adducts to DNA and induction of cancer, it is necessary to identify the ultimate metabolite and to determine the base specificity of nucleotides involved in binding.

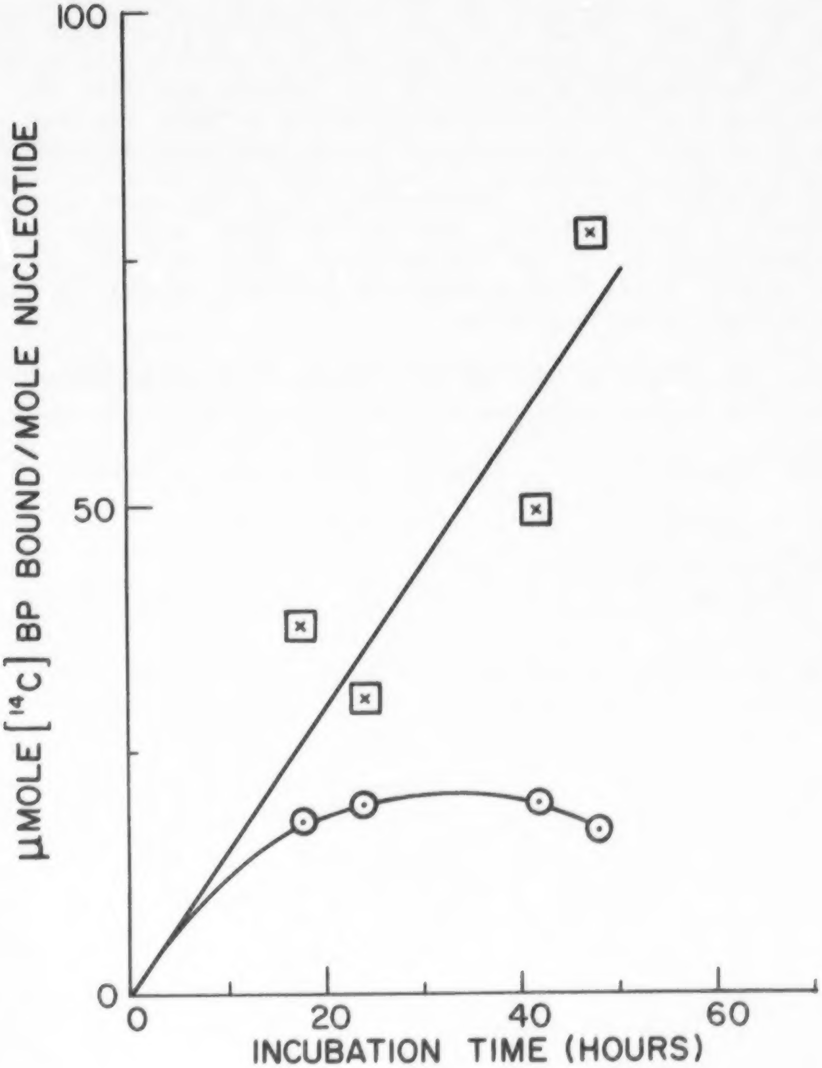


Figure 4. Time course of binding of (^{14}C)BaP to DNA and RNA in confluent HEC cultures. HEC DNA, ⊙—⊙ ; HEC RNA, ⊠———⊠. The results represent mean values obtained from five independent studies.

The detection by standard analytical techniques of the reactive metabolites *in vivo* has been hindered for several reasons: (1) the large number of metabolites produced; (2) the very low levels of reaction with nucleic acids (1/10,000 - 1/1000,000), and (3) the structural complexity of the nucleoside adducts.

Identification of an Ultimate Metabolite of Benzo(a)pyrene

Some of the multiple products of BaP metabolism are illustrated in Figure 3. The AHH enzyme system oxidizes BaP to a variety of quinones, phenols, dihydrodials and epoxides (Selkirk *et al*., 1974, 1976). Boyland (1950) was the first to propose that arene oxide derivatives of carcinogenic PAH molecules are the reactive intermediates responsible for the *in vivo* binding of the parent hydrocarbon to nucleic acids. The properties of synthetic K-region epoxides lent some support to this hypothesis. They are alkylating agents that react covalently with nucleic acids (Grover *et al*., 1972): they are mutagenic in several systems and can induce malignant transformation of rodent cells in culture (Heidelberger, 1974; Marquardt *et al*., 1974). Studies during the late 1960s until the mid-1970s tended to favor, therefore, the K-region of BaP (BaP-4,5-oxide, see Figure 4) as the important reactive metabolite of this hydrocarbon (Grover *et al*., 1972; Heidelberger, 1974).

The first technique successfully utilized for identification of the covalently bound form of BaP involved enzymatic degradation, to the deoxynucleoside level, of the modified DNA obtained from cells exposed to radioactive BaP. These products were co-chromatographed on a Sephadex LH-20 column, which separates materials on the basis of hydrophobicity, with UV markers of DNA-bound model compounds synthesized *in vitro*. Using this approach, Baird (1975) established that the digest of DNA reacted *in vitro* with BP-4,5-oxide did not co-chromatograph with *in vivo* products (Jeffrey *et al*., 1976). At the same time, it was demonstrated that when BaP and a series of BaP metabolites were added to a microsomal system in the presence of DNA, BaP 7,8-dihydrodiol (see Figure 3) was the most active compound in terms of covalent binding (Borgen *et al*., 1973). The latter compound bound to DNA to a tenfold greater extent than BaP, suggesting that it is a proximate carcinogen which is converted by the microsomes to an ultimate carcinogen. One year later, Sims and co-workers (1974) extended this result by providing evidence for a two-stage metabolic activation of BaP. This initially involves formation of BaP 7,8-dihydrodiol, which then is further metabolized on the 9,10 ring positions to give BaP 7,8-dihydrodiol 9,10-oxide (BaPDE).

In addition to column chromatography of enzymatic digests, fluorescence spectroscopy is an extremely useful technique for detecting and identifying BaP adducts in nucleic acids (Ivanovic *et al*., 1976). We have used this procedure at liquid nitrogen temperature which increases the sensitivity about tenfold (Ivanovic *et al*., 1976). Fluorescence measurements were utilized to obtain information regarding the structure of the DNA- and RNA-bound chromophore formed in HEC at different time points. As illustrated in Figure 5, the fluorescence emission spectra of DNA and RNA samples obtained from HEC following an 18-hr incubation with the parent hydrocarbon closely resembled those obtained with DNA or RNA reacted with BaPDE *in vitro*. The fluorescence spectra of the 24- and 42-hr DNA and RNA samples (Figure 5) and the 48-hr sample (not shown here) were also qualitatively similar to the 18-hr samples. These studies provided fluorescence spectral evidence that BaPDE is the ultimate metabolite of BaP responsible for covalent binding to DNA and RNA.

Two stereoisomers of BaPDE have been systhesized (Figure 6). In isomer I, sometimes called *anti*, the 7-hydroxyl and 9,10-oxide groups are on opposite sides of the plane of the ring system. In isomer II, also called *syn*, the 7-hydroxyl and 9,10-oxide groups are on the same side. Depending on the location of the 7-hydroxyl group (whether above or below the plane), we can further distinguish two enantiomers for each stereoisomer. As presented in Figure 7, in the 7R enantiomer of BaPDE I the 7-hydroxyl group is above the plane, whereas in its enantiomeric pair, 7S BaPDE I, the 7-hydroxyl group is below the plane. Similarly, there are two enantiomers for isomer II. In 7R BaPDE II, the 7-hydroxyl is above, and in 7S BaPDE II it is below the plane of the ring system. An additional complexity is introduced by the nature of the 9,10-oxide ring opening, which can be *cis* or *trans*, in the reaction with base residues in nucleic acids.

Base Specificity of Benzo(a)pyrene-Nucleic Acid Binding In Vivo

Fluorescence spectroscopy does not distinguish between either the different BaPDE isomers or different nucleosides involved in nucleic acid binding. To obtain information on these compounds, it was necessary to use high pressure liquid chromatography (HPLC) analysis of the nucleoside adducts.

Benzo(a)pyrene-RNA Adducts in Hamster Embryo Cells--

RNA samples obtained from HEC cultures exposed to (^{14}C)BaP for either 18 or 42 hr (see Figure 3) were hydrolyzed with KOH (Ivanovic *et al*., 1978) and BaP modified nucleosides were analyzed utilizing various BaPDE nucleoside adducts synthesized *in vitro* as markers.. Figure 8A illustrates the elution positions of several of these markers, whose structures and stereochemistry have been previously elucidated by NMR, mass spectroscopy, and circular dichroism (Moore *et al*., 1977; Jeffrey *et al*., 1976; 1977; Weinstein *et al*., 1976). All of the peaks are guanosine adducts resulting from addition of the 2-amino group of guanosine to the 10 position of BaPDE. The first peak in Figure 8A, GI-1, results from *trans* opening of 7S BaPDE I, whereas the last peak, GI-3, represents its enantiomeric pair derived from 7R BaPDE I. Peak GI-2 (Figure 8A) is analogous to GI-3 but is formed by *cis* addition. GII-1 marker in Figure 8A is a result of *trans* opening of 7S BaPDE II (Moore *et al*., 1977; Jeffrey *et al*., 1977). Figures 8B and 8C represent RNA digests obtained from cells incubated for either 18 or 42 hr with BaP. The 18-hr time point HPLC

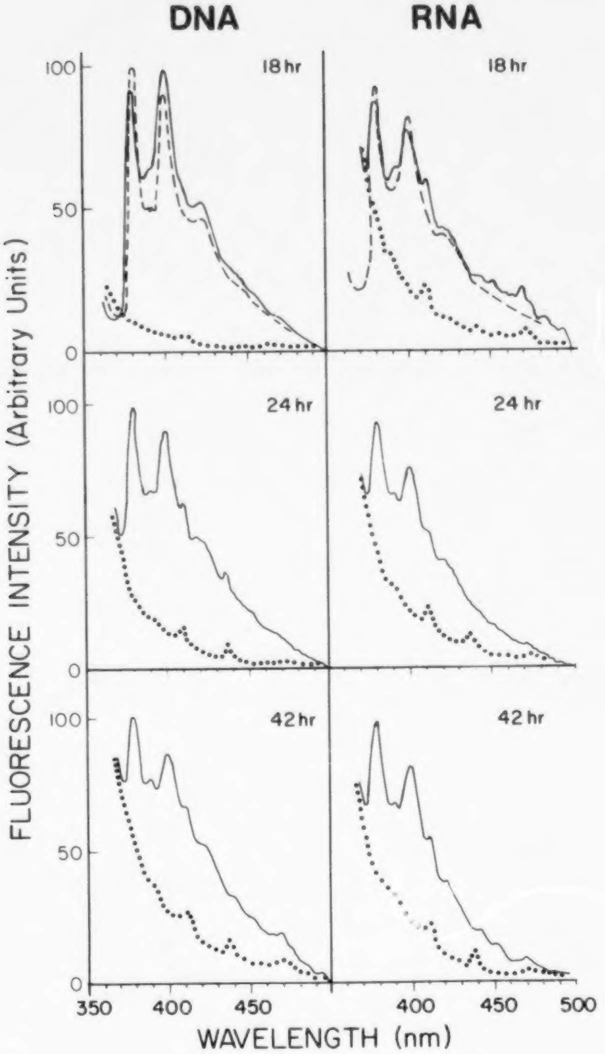
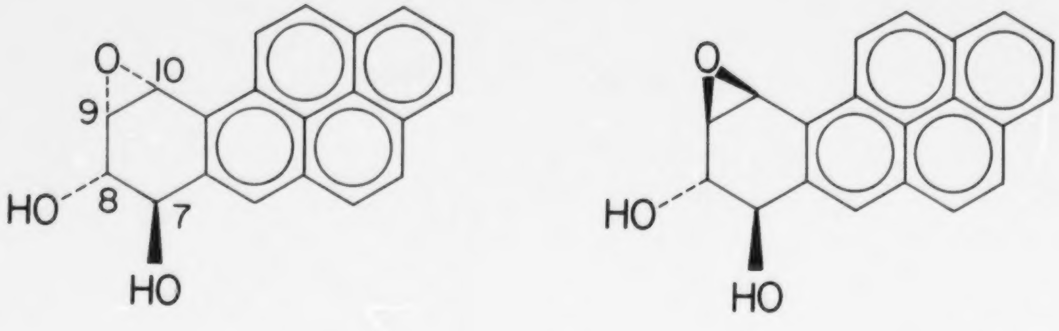
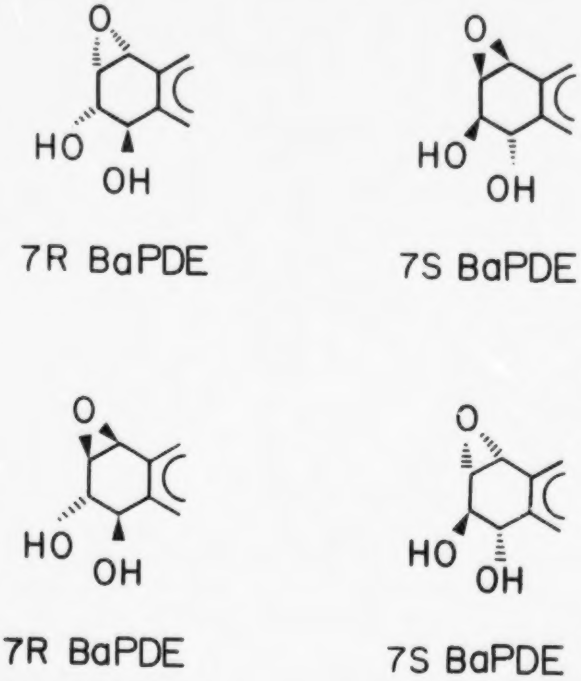


Figure 5. Low temperature fluorescence emission spectra of DNA and RNA obtained from HEC cultures exposed to (^{14}C)BaP for 18, 24, or 42 hr, with comparisons to *in vitro* modified DNA. *In vivo* modified nucleic acid samples, (-----); control NA, (.....); DNA of RNA modified *in vitro* with BaPDE (-----).



I II

Figure 6. Stereochemical structures of two isomers of BaPDE.



7R BaPDE 7S BaPDE

7R BaPDE 7S BaPDE

Figure 7. Structures of enantiomers of BaPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, and BaPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene. 7R BaPDE I, the 7R,8S,9R,10R enantiomer of BaPDE 1 [or (±) BaPDE I, derived from (-) BaP 7,8-dihydrodiol]; 7S BaPDE I, 7S,8R,9S,10S enantiomer of BaPDE I [or (-) BaPDE I, derived from (+) BaP 7,8-dihydrodiol]; 7S BaPDE II, 7S,8R,9R,10R enantiomer of BaPDE II [or (+) BaPDE II derived from (+) BaP 7,8-dihydrodiol].

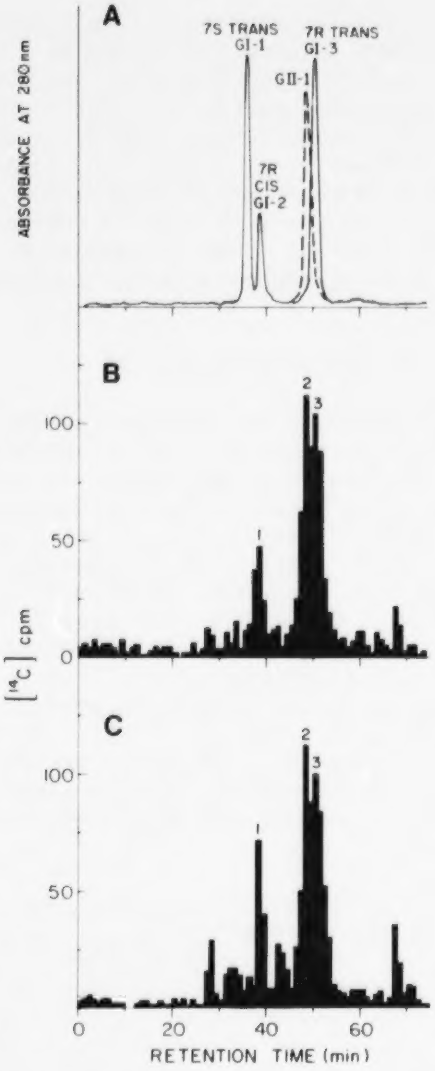


Figure 8. HPLC profiles of RNA adducts formed in confluent HEC cultures incubated with (^{14}C)BaP.

A. *In vitro* markers: Elution positions of guanosine adducts formed by *in vitro* reactions with BaPDE I ("GI-1,2,3") with designation of the enantiomeric form and character of 9,10-oxide ring opening and of a guanosine adduct formed with BaPDE II ("GII-I"). For a further description of these markers, see text.

B. RNA isolated from HEC after 18 hr exposure of the culture to (^{14}C)BaP.

C. RNA isolated from HEC after 42 hr exposure of the culture to (^{13}C)BaP.

profile (Figure 8B) reveals three major radioactive peaks designated 1-3. Comparison with *in vitro* markers (Figure 8A) indicated that HEC RNA peak 1 co-chromatographed with the *cis* 7R BaPDE I adduct. HEC RNA peak 2 elutes with the *trans* 7S BaPDE II guanosine marker and peak 3 with *trans* 7R BaPDE I. Similar BaP-bound RNA adducts have been detected in human and bovine bronchial explants (Jeffrey *et al*., 1977) and mouse skin RNA (Moore *et al*., 1977). The 42 hr time point profile (Figure 8C) is qualitatively similar to the 18 hr profile with the exception of increased prominence of certain minor peaks. Their retention times correspond to previously described BaPDE-cytidine and BaPDE-adenosine adducts (Jennette *et al*., 1977).

Benzo(a)pyrene-DNA Adducts in Hamster Embryo Cells--

Figure 9B presents the BaP-modified deoxynucleosides obtained after enzymatic digestion of HEC DNA obtained from cells incubated with BaP (^{14}C) for 21 hr. The HPLC profile revealed 4 major radioactive peaks, designated HEC DNA-1-4, and a few minor products. Comparison of these products with BaPDE marker adducts synthesized *in vitro* (Figure 9A) led to the following assignments. Three peaks (2-4, Figure 9B) are BaPDE-deoxyguanosine adducts resulting from addition of the two amino group of guanine to the 10 position of BaPDE. HEC DNA peak 2 (Figure 9B) coincides in its elution position with the deoxyguanosine-*trans* 7S BaPDE I (Figure 9A) and peak 3 with its enantiomeric pair from *trans* 7R BaPDE I. The latter product was detected as the single BaP product in human and bovine bronchial explant DNA (Jeffrey *et al*., 1977). HEC DNA peak 4 corresponds to a deoxyguanosine-BaPDE II product. HEC DNA I in Figure 9B elutes in the region of deoxycytidine-BaPDE adducts (Jennette *et al*., 1977) and peaks 5-7 cochromatograph with multiple deoxyadenosine-BaPDE adducts (dAI-1-4 in Figure 9A).

Figure 9C illustrates the effect of "post treatment incubation" on HPLC profiles. In this type of experiment cells were exposed to (^{14}C)BaP for 21 hr. Following this, the radioactive medium was removed, the cell monolayer rinsed twice with warmed medium and the cells were then incubated in BaP-free medium for an additional 24 hr. The removal of carcinogen from the medium was associated with a 40% reduction (Ivanovic *et al*., 1978) in the amount of BaP (^{14}C) bound to DNA when compared to the plateau value (see Figure 4) seen when carcinogen was not removed. This indicates that a DNA repair system that excises the BaP residues from the DNA is active in hamster embryo cells. The HPLC profile (Figure 9C) of a DNA digest of the latter sample shows that, in addition to reduction in the total amount of BaP adducts, there is also a change in the relative contributions of individual peaks to the total profile. Although the changes are complex, perhaps the most striking one is a decrease in the relative abundance of peak 3 and an increase of peak 2. This suggests that the peak 3 adduct is excised at a more rapid rate than peak 2. Other peaks may also undergo differential rates of excision, but this requires further study.

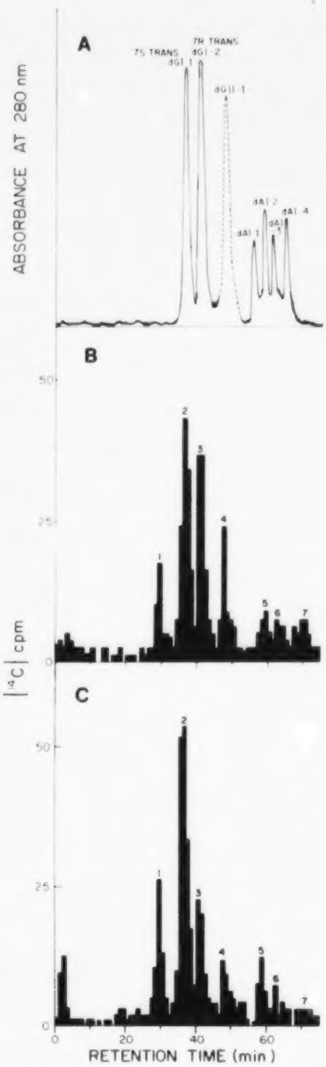


Figure 9. HPLC profiles of DNA adducts formed in confluent HEC cultures incubated with (^{14}C)BaP.

A. *In vitro* markers: Elution position of deoxyguanosine and deoxyadenosine adducts formed by *in vitro* reactions with BaPDE I ("dGI-1,2" with designation of the enantiomeric form and character of 9,10-oxide ring opening; and "dA-1,2,3,4") and of a deoxyguanosine adduct formed with BaPDE II ("dGII-I"). For a further description of these adducts, see text.

B. DNA isolated from HEC after 21 hr exposure of the culture to (^{14}C)BaP.

C. DNA isolated from a culture of HEC exposed to (^{14}C)BaP for 21 hr and then incubated for additional 24 hr in the absence of BaP ("Post-treatment Incubation").

It has been reported that hamster embryo cultures are readily transformed by BaP (Berwald and Sachs, 1965; DePaolo and Donovon, 1967) and even better by BaPDE (Mager *et al*., 1977). On the other hand, at the present time it has not been established that the exposure of normal human cell cultures to BaP results in reproducible transformation. A comparison of BaP adducts in rodent and human cell cultures is presented in Table 2.

TABLE 2. COMPARISON OF BENZO(A)PYRENE-RNA AND -DNA ADDUCTS FORMED IN CULTURE

	Hamster Embryo Cells (Confluent, Primary Cultures) (Ivanovic *et al*., 1978)	Human and Bovine Bronchial Segments (Jeffrey *et al*., 1977)
RNA	7R BaPDE I - (Cis) - G	7R BaPDE I - (Cis) - G
		BaPDe I - C
	7S BaPDE II - (Trans)-G	7S BaPDE II-(Trans)-G
	7R BaPDE I - (Trans)-G	7R BaPDE I -(Trans)-G
DNA	BaPDE - dC?	
	7S BaPDE I-(Trans)-dG	
	7R BaPDE I-(Trans)-dG	7R BaPDE I -(Trans)-dG
	BaPDE II-dG	
	BaPDE - dA	

RNA adducts in hamster embryo cells have a HPLC profile which is very similar to that of human and bovine (Jeffrey *et al*., 1977), as well as mouse skin RNA (Moore *et al*., 1977). On the other hand, the multiple BaP products in DNA of hamster embryo culture are in contrast to the much simpler profile in DNA of human and bovine bronchial explants in which the predominant product is the 7R BaPDE I-deoxyguanosine adduct (Jeffrey *et al*., 1977).

Additional variations in profiles of BaPDE-DNA adducts have also been seen in the mouse fibroblast 10 T ½cell line (Brown et al., 1979).

DISCUSSION

It appears, therefore, that although in all cases studied so far BaPDE is the major BaP metabolite responsible for covalent binding to RNA and DNA in mammalian cells, there are major differences between species and cell types in terms of the relative abundance of the different types of deoxynucleoside adducts that are formed. The relative importance of the individual adducts with respect to the carcinogenic process is not known at the present time.

To our knowledge, detailed studies similar to those described above have not been performed with marine organisms. There is some evidence that DNA-BaP adducts produced by fish liver microsomes, are primarily derived from the BaPDE metabolite of BaP (Ahokas,J.T., unpublished studies). Further studies on BaP metabolism in diverse marine organisms is of considerable interest, particularly in relation to cancer induction in these organisms. Such studies may provide information about the role of various BaP metabolites in the induction of neoplasia in fish and other marine organisms. Since, however, the pathways involved in BaP metabolism are used for detoxificatio purposes, the fact that marine organisms metabolize BaP and related compounds to proximate and ultimate carcinogens does not mean that these reactions occurring in marine organisms represent an immediate danger to humans. The diol epoxides have very short half-lives in aqueous solution. For example, Yagi and co-workers (1977) have reported that in tissue culture medium BaPDE I has a half-life of 8 min, and BaPDE II has a half-life of only 0.5 min. It is unlikely, therefore, that humans would suffer hazardous exposure to ultimate carcinogens formed by marine organisms. Once such ultimate metabolites are bound to the DNA and other cellular macromolecules of marine organisms, it is also highly unlikely that subsequent ingestion of these BaP-modified macromolecules by humans would be hazardous. In contrast, bioaccumulation of the parent compound in tissues of marine organisms could conceivably represent a hazard to humans when such material is ingested.

The ultimate carcinogenic metabolites produced by marine organisms, however, could lead to mutations, developmental defects, neoplasia, or lethal effects within these species. The possible extinction of certain marine organisms that suffer from these toxic effects could disturb marine ecosystems and thus indirectly be harmful to the human environment. At the same time, it may be useful to monitor the occurrence of tumors in marine organisms to provide an index of the presence of potential human carcinogens in the marine environment. The validity of this surveillance system will depend, in part, on knowing the comparative similarities and differences in metabolism of BaP and other potential carcinogens between humans and the marine organisms being studied. This provides an additional reason for encouraging more detailed studies on BaP metabolism and binding to cellular macromolecules in marine organisms.

ACKNOWLEDGMENTS

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POLYCYCLIC AROMATIC HYDROCARBONS IN THE AQUATIC ENVIRONMENT AND CANCER RISK TO AQUATIC ORGANISMS AND MAN

by

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ABSTRACT

Published values for the concentration of polycyclic aromatic hydrocarbons (PAHs), and in particular benzo(a)pyrene (BaP), in fresh and marine waters and in the tissues of aquatic animals are reviewed. All but the most heavily contaminated fresh waters contain total PAH concentrations in the parts per trillion or low parts per billion range. The limited data for marine waters indicate similar concentrations. Aquatic animals generally contain 0 to 50 μg BaP/kg dry weight. Heavily contaminated animals may contain up to 5000 μg BaP/kg. Teratogenic and/or carcinogenic responses have been induced in sponges, planaria, echinoderm larvae, teleost fish, and amphibians by exposure to carcinogenic PAHs. There are many reports of high incidences of cancer-like growths in natural populations of aquatic animals and plants. In most cases the causative agent or agents are uknown. Circumstantial evidence implicating PAHs in these natural outbreaks of cancers has been provided in only a few cases. It is estimated that less than 0.1% of the PAH ingested by man comes from drinking water. Fishery products consumed by man contain PAH concentrations similar to those in smoked and charcoal-broiled meats and green vegetables. Aquatic PAHs represent a minor source of PAH in the human environment.

INTRODUCTION

It has been recognized for many years that some polycyclic aromatic hydrocarbons (PAHs) can cause cancer in laboratory mammals and possibly man. Correlations have been made between occupational or other exposure to PAH and the incidence of human cancer (IARC, 1973; NAS, 1972; Bridboard *et al*., 1976). It is now generally agreed that metabolic activation by the mixed function oxygenase (MFO) system and sometimes by epoxide hydrase is a necessary prerequisite for PAH-induced carcinogenesis and mutagenesis (Jerina and Daly, 1974; Huberman *et al*., 1976; DePierre and Ernster, 1978). Many PAH do not yield carcinogenic metabolites, and only certain metabolites of carcinogenic PAH are carcinogenic. For instance, more than two dozen metabolites of benzo(a)pyrene (BaP) have been identified in mammalian systems (DePierre and Ernster, 1978). Yet most of the carcinogenicity of BaP is thought to be due to the isomeric 9,10-epoxy-7,8-dihydro-7,8-dihydroxybenzo(a)pyrenes (Sims _et al_., 1974; Lehr and Jerina, 1977; Yang _et al_., 1977, 1978).

The relative carcinogenicity of several PAHs are listed in Table 1. 4-, 5-, and 6-Ring PAH are more carcinogenic than either smaller or larger ring systems; highly angular configurations are more carcinogenic than either linear or highly condensed ring systems. Alkylation may substantially modify carcinogenicity of a PAH. Position of alkyl substituents is extremely important. The degree of carcinogenicity of a PAH is related to structure and reactivity of its major metabolites. Newman (1976) compared the carcinogenic activity of 12 monomethylbenz(a)-anthracenes. 7-methylbenz(a)anthracene was most active, 6-,8-, and 12-methyl isomers were slightly active, and the remaining monomethylbenz(a)-anthracenes were inactive. The author concluded that the 7-position in benz(a)anthracene is the position at which detoxification occurs.

TABLE 1. RELATIVE CARCINOGENICITY OF PAH TO LABORATORY MAMMALS (FROM NAS, 1972)

Compound	Carginogenicity	Compound	Carginogenicity[1]
Anthracene	-	Aceanthrylene	-
Phenanthrene	-	Benz(j)aceanthrylene	++
Benz(a)anthracene	+	= cholanthrene	
7,12-Dimethylbenz(a)anthracene	++++	3-Methylcholanthrene	++++
Dibenz(aj)anthracene	+	Naphthacene	-
Dibenz(ah)anthracene	+++	Pyrene	-
Dibenz(ac)anthracene	+	Benzo(a)pyrene	+++
Benzo(a)phenanthrene	+++	Benzo(e)pyrene	-
Fluorene	-	Dibenzo(al)pyrene	±
Benzo(a)fluorene	-	Dibenzo(ah)pyrene	+++
Benzo(b)fluorene	-	Dibenzo(ai)pyrene	+++
Benzo(c)fluorene	-	Dibenzo(cd,jk)pyrene	-
Dibenzo(ag)fluorene	+	Indeno(1,2,3-cd)pyrene	+
Dibenzo(ah)fluorene	±	Chrysene	±
Dibenzo(ac)fluorene	±	Dibenzo(b,def)chrysene	++
Fluoranthene	-	Dibenzo(def,p)chrysene	+
Benzo(b)fluoranthene	++	Dibenzo(def,mno)chrysene	-
Benzo(j)fluoranthene	++	= anthanthrene	
Benzo(k)fluoranthene	-	Perylene	-
Benzo(mno)fluoranthene	-	Benzo(ghi)perylene	-
		Coronene	-

* -, not carcinogenic; ± uncertain or weakly carcinogenic; +, carcinogenic ++, +++, ++++, strongly carcinogenic

The 7-position must be blocked to obtain significant carcinogenic activity. The 5-position is that at which metabolism occurs to produce cancer. Blockage of this position destroys carcinogenic activity. Similar results were obtained with methylchrysenes (Hecht *et al*., 1976). 5-methylchrysene demonstrated very high carcinogenic activity, equal to or greater than that of BaP. 2-methylchrysene had moderate activity; other methylchrysenes were inactive. Thus, alkylation affects carcinogenicity of PAH by altering the position of initial enzymatic attack on the PAH molecule by the MFO-cytochrome P-450 system.

Different species of organisms vary substantially in sensitivity to PAH-induced carcinogenesis. This may result from interspecific differences in the levels of MFO-cytochrome P-450 and epoxide hydrase activity, sterochemistry of the reactions catalyzed by enzymes from different species, and rate at which the active metabolites are converted to less active products. Several species of aquatic annelids, arthropods, fish, and amphibians possess the requisite enzyme systems for metabolic activation of PAH (Neff, 1978). However, it is uncertain in most cases whether these enzymes produce the same metabolites as those produced by mammalian enzymes. The limited data available indicate that some aquatic animals do produce the active metabolites necessary for carcinogenesis (e.g., the 5,6-dihydrodiol of benz(a)anthracene and the 7,8-dihydrodiol of benzo(a)-pyrene).

Stegeman (1977) provided evidence that the MFO-cytochrome P-450 system of fish is able to produce carcinogenic or mutagenic metabolites, at least *in vitro*. He added bacteria *Salmonella typhimurium* strain TA-98 to a reaction mixture containing (BaP) and MFO enzymes from scup, *Stenotomus versicolor*, liver. After a suitable incubation period, the number of bacterial survivors was reduced and the number of bacterial mutants increased (Table 2), indicating that toxic and mutagenic metabolites had been produced by the fish liver enzymes. Similar results were obtained by Payne *et al*. (1978) with hepatic microsomes of rainbow trout, *Salmo gairdneri*, and a PAH-enriched fraction of used crankcase oil. Thus, both marine and freshwater fish are able to produce mutagenic and, by inference, carcinogenic metabolites from PAHs.

Laboratory Studies

Many, but not all, carcinogenic chemicals are also mutagenic or teratogenic. That is, they can cause abnormal growth in organisms exposed to the chemical or birth defects in the offspring of exposed parents. Thus, the carcinogenicity of a chemical is often inferred from its mutagenicity or teratogenicity. Some sublethal effects of PAHs, suggestive of mutagenesis or teratogenesis, have been described in marine organisms; e.g., growth stimulation in algae (Boney and Corner, 1962; Boney, 1974) and abnormal development of sea urchin embryos (Ceas, 1974; de Angelis and Giordano, 1974).

TABLE 2. BIOACTIVATION OF BENZO(A)PYRENE TO TOXIC AND MUTAGENIC DERIVATIONS BY SCUP, STENOTOMUS VERSICOLOR, LIVER MIXED FUNCTION OXYGENASES. SCUP LIVER MICROSOMES WERE INCUBATED WITH SALMONELLA TYPHEMURIUM STRAIN TA-98 AND APPROPRIATE COFACTORS (FROM STEGEMAN, 1977)

Incubation Conditions	Bacterial Surviyors ($x10^7$)	Mutant Fraction* ($x10^{-8}$)
Complete (12.5 μg BaP/mℓ)	53	47.2
Minus BaP	1088	0.55
Minus NADPH	945	0.21

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* Mutant fraction refers to the number of His$^+$ revertants per number of survivors.

Unfortunately, with the exception of work with amphibians, there has been relatively little laboratory research on the carcinogenicity, mutagenicity, and teratogenicity of PAH in aquatic organisms. Exposure of colonial calcareous sponges, Leucosolenia complicata and L. variabilis, to 5 g/ℓ BaP resulted in choanocyte damage and abnormal growth of the oscular tube (Korotkova and Tokin, 1968). The solitary sponge, Sycon raphanus, was unaffected.

Foster (1969) exposed adult planarians, Dugesia dorotocephala, to either 3-methylcholanthrene or BaP during regeneration of excised body parts; 9% of the planaria exposed to 3-methylcholanthrene and 7% of those exposed to BaP developed lethal papilliform tumors and other malformations. He suggested that the malformations and possibly also the tumors were derived from stimulated regenerative cells--totipotent neoblasts. Offspring of surviving PAH-exposed adults developed abnormal growths similar to those of the exposed adults. 3-methylcholanthrene was more carcinogenic and teratogenic than BaP to the offspring (Table 3). It is tempting to speculate, based on these results, either that planaria possess the MFO-cytochrome P-450 system or that PAH, which accumulated in their transparent tissues, were photooxidized to mutagenic products.

TABLE 3. LETHAL TUMORS AND DEVELOPMENTAL ANOMALIES IN OFFSPRING OF PLANARIA, DUGESIA DOROTOCEPHALA, TREATED WITH 3-METHYLCHOLANTHRENE OR BENZO(A)PYRENE (FROM FOSTER, 1969)

Treatment of parents	Number of offspring	Tumors in offspring	Malformations in offspring
3-Methylcholanthrene (regenerated)	40	12 (papilliform tumors)	6 eyespots poorly developed; 4 enlarged heads and eyes; 3 fused eyespots
Benzo(a)pyrene (regenerated)	75	2 (nodular tumors)	3 enlarged heads and eyes 2 small heads and eyes 2 fused eyespots
Acetone control (regenerated)	65	1 (nodular tumor)	variation in pigmentation 2 enlarged heads and eyes
Untreated controls (not regenerated)	77	none	1 small head and eyes 1 fused eyespot

Khudolei and Sirenko (1977) induced the formation of neoplasms in the digestive gland and hematopoietic system of fresh water mussels, Unio pictorum, by subjecting the bivalves to 200 to 400 parts per million (ppm) diethyl- and dimethylnitrosamines in water. However, there are no published reports of cancer induction in bivalve molluscs by exposure to or injection of PAHs. Such an investigation would be very informative for several reasons. The majority of bivalve molluscs studied to date lack or have very low MFO activity and therefore should not be able to activate PAH to carcinogenic metabolites. Yet there are many reports of natural populations of bivalves with high incidences of neoplastic disease. Some of these populations are from oil-polluted habitats. If bivalves are not sensitive to PAH-induced carcinogenesis, another causative agent for these outbreaks of neoplasia must be sought. If PAH can cause cancer in bivalves, a closer look at how this is accomplished may lead to new insights into the mechanisms of PAH-induced carcinogenesis.

Bourne and Jones (1973) exposed cell cultures derived from fibroblastic gonad cells of rainbow trout, Salmo gairdneri (RTG-2), and epithelioid cells of fathead minnows, Pimephales promelas (FHM), to 7, 12-dimethylbenz(a)anthracene. The PAH contaminant inhibited mitosis and induced an increase in the number of multinucleate cells. RTG-2 cells were more sensitive than FHM cells to the inhibition of mitosis, although FHM cells showed a much greater incidence of multinucleate cells than did RTG-2 cells. In FHM cultures many cells were extremely abnormal with a reduction in cytoplasm, clumped chromation, and basophilic cytoplasm. These results suggest that the fish cells were converting 7,12-dimethylbenz(a)anthracene to reactive metabolites which bonded chemically to nuclear proteins and DNA.

Zebra fish embryos, Brachydanio rerio, exposed to 0.56 ppm 7,12-dimethylbenz(a)anthracene developed tail necrosis, tumor-like growths, an enlarged pericardium, and shortened body (Jones and Huffman, 1957). Epithelial cells and nuclei of PAH-exposed embryos were enlarged and granular. The growth rate of some parts of the embryos was accelerated. Ermer (1970) painted 0.5 mg of 3-methylcholanthrene twice a week for 3 to 6 months on the skin of three freshwater fish: three-spined stickleback, Gasterosteus aculetus; bitterling, Rhodeus amarus; and carp, Cyprinus carpio. This treatment produced epitheliomas (skin cancer) in G. aculetus and R. amarus but not in C. carpio. Similar results were produced when BaP was used. Epitheliomas were induced in the first two but not the third fish. He also injected G. aculetus with 0.1 mℓ of 1% BaP in glycerol ten times (10 mg BaP) and maintained the fish for four months. BaP produced injection-site necrosis but no neoplasms.

Neukomn (1974) induced cancerous lesions in newts, Triturus cristatus, by subcutaneous injection of several PAH into regenerative areas of the epidermis. Epithelial hyperplasia was followed by infiltration of underlying tissues. Neoplastic infiltration occurred within four to five days after injection; activity continued for 12 to 20 days and culminated in a diffuse tumor. The PAH tested varied in their carcinogenicity to the newt in the following order of decreasing potency: dibenz(ah)anthracene, 3-methylcholanthrene, BaP, and 9,10-dimethylbenz(a)thracene. Chrysene and benz(a)anthracene were weakly active; pyrene was inactive.

Seilern-Aspang and Kratochwil (1962, 1963) induced epitheliomas in newts, Triturus cristatus, by subcutaneous injection of BaP. The cancer often showed regression and differentiation into non-malignant tissue when a regeneration process was initiated in the animals by amputating a limb or the tail. Pizzarello and Wolsky (1966) showed that if regeneration was initiated before injection of 3-methylcholanthrene or dibenz(ah)anthracene into T. viridescans, the cancer did not form. However, the presence of carcinogenic PAH in the animals retarded the process of regeneration. These results suggest that regeneration and carcinogenesis are antagonistic processes in the newts. Regeneration inhibits malignant growth, but the presence of carcinogenic tendencies in the animal during regeneration retards reconstruction of tissues lost by amputation.

Crystals of 3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, or BaP were implanted subcutaneously in the tail of toad tadpoles, Bufo arenarum, (de Lustig and Matos, 1971). After a few days papillomas and lymphomas were detected in 90% of the treated animals. Tumor-like cells invaded normal structures in the tail, displacing the newly formed tissues. Several of the PAH-treated tadpoles developed an accessory tail or notochord. Fluorene and paraffin were completely inactive in inducing cancer or accessory body structures. Subsequently, Matos and de Lustig (1973) showed that if the tail of the tadpole was amputated at the center of the treated area nine days after implantation of PAH crystals to promote the formation of a regenerative blastema, the PAH-induced cancerous nodules were encapsulated and eventually destroyed. Incidence of PAH induced teratogenic effects, including supernumerary fins and accessory notochords, was increased by a regenerative field produced by tail amputation. 7,12-dimethylbenz(a)anthracene was substantially more teratogenic than either 3-methylcholanthrene or BaP. In a similar investigation, Ruben and Balls (1964) induced lymphosarcomas in the African clawed toad, *Xenopus laevis*, by implantation of 3-methlcholanthrene into the forelimbs or abdomen. The presence of a regenerating limb system in the carcinogenic environment did not diminish carcinogenic activity. Accessory limb structures were obtained near the sites of crystal implantation in some cases.

These studies show that carcinogenic PAHs can produce cancer-like growths and cause teratogenesis and mutagenesis in some aquatic invertebrates and vertebrates. The number of species examined to date is very low, however, and there are no reports of induction of cancer by exposure of aquatic animals to environmentally realistic levels of carcinogenic PAHs in the water, food, or sediments.

Field Studies

Published literature on the incidence of cancer or cancer-like growths in tissues of natural populations of marine and freshwater invertebratres and fish is growing rapidly. Much of the recent literature is reviewed in several papers from two recent symposium volumes (Dawe *et al*., 1976; Kraybill *et al*., 1977). In many cases organisms from polluted environments have a higher incidence of tumors and hyperplastic diseases than those from uncontaminated environments. In the vast majority of cases the causative agent or agents are unknown. A great many different inorganic and organic chemicals can induce cancer in sensitive species and polluted aquatic habitats nearly always contain a wide variety of potential carcinogens (Bergel, 1974). Fish are known to be susceptible to tumor induction by chemical gents, ionizing radiation, physical factors, and viruses (Stich and Acton, 1976). In addition, fish neoplasms can be genetically induced. To date, carcinogenic PAHs have not been unequivocally identified as the causative agent for an increased incidence of cancer in any natural population of aquatic organisms.

There are several reports of increased incidence of cancerous growths in aquatic animals in the vicinity of an oil spill (Hodgins *et al*., 1977). Yevich and Barszcz (1977) reported a high incidence of gonadal neoplasms in soft-shell clams, *Mya arenaria*, from Long Cove, Searsport, ME, and of hematopoietic neoplasms in the same species from Harpswell, Neck, ME. Although both sites had been contaminated with refined oil, the authors concluded that they could not say that oil was in any way a causative factor in inducing the neoplasms.

Powell et al. (1970) induced hyperplasia of ovicells in the estuarine bryozoan, Schizoporella unicorns, by placing normal colonies in close proximity to coal-tar derivatives in an estuary. The authors attributed uncontrolled growth of the reproductive structures to stimulation by several PAH known to be present in coal tar. However, Straughan and Lawrence (1975) found no ovicell hyperplasia in bryozoans from surface, subsurface, and benthic kelp fronds in the vicinity of Coal Oil Point, CA, an area of chronic submarine oil seepage.

Brown et al. (1973, 1977) reported a significantly higher frequency of tumors in 2121 freshwater fish from the polluted Fox River, IL, than in 4539 fish from relatively unpolluted Lake of the Woods, Ontario, Canada. In fish from the Fox River, incidence of neoplasia ranged from 1.17 to 12.2% in different species with a mean of 4.38%. Fish from the unpolluted habitat had a mean incidence of neoplasia of 1.03% with a range of 0 to 2.56% in different species. The brown bullhead, Ictalurus nebulosus, was the most seriously infected fish in the Fox River sample. Several organic and inorganic pollutants were detected at higher concentrations in the Fox River than in the Lake of the Woods. Concentrations of benzene, toluene, naphthalene, and benzanthracene in the Fox River in 1976 were 0.2, 0.1, 0.2, and 0.05 ppm, respectively. These chemicals were not detected in Canadian waters. Several chlorinated hydrocarbons and heavy metals were also present at higher concentrations in the Fox River than in the Lake of the Woods. Some of the tumors were apparently caused by viruses, but the etiology of other tumors is still unknown.

A stronger case can be made for implicating PAH as the causative agent of a high incidence of neoplasia in tiger salamanders, Ambystoma tigrinum, from a 13 hectacre sewage effluent lagoon at Reese Air Force Base, TX (Rose, 1976; 1977). Here, the incidence of neoplastic and non-neoplastic lesions in neotenic salamanders reached a maximum of 53% in 1975. Water and sediment of the lagoon were analyzed for organochlorine and organophosphate pesticides, nitrosamines, several heavy metals, and PAH. All but the PAH were below the limits of detection or were present at normal expected concentrations. Lagoon water contained 0.085 $\mu g/\ell$ BaP, and sludge on the floor of the lagoon contained high concentrations of several PAH, especially perylene (Table 4). The sewage effluent lagoon is quite eutrophic and contains frequent large blooms of phytoplankton, daphnia, and copepods. Dead planktonic organisms accumulate in large mats on the anoxic bottom sludge. These are ideal conditions for indirect biosynthesis of PAH by reduction of extended quinones of biological origin or of direct biosynthesis by anaerobic bacteria in the sludge. The high concentration of perylene supports the former hypothesis. Other sources of this unusual PAH assemblage are also possible.

Salamanders from the lagoon had elevated hepatic MFO activity. MFO activity in laboratory-reared salamanders could be induced to a level similar to that in lagoon animals by exposing them to 3-methylcholanthrene in water (Busbee et al., 1975, 1978). Other inducers of MFO activity--organochlorine and organophosphate insecticides--were absent from lagoon water. All these observations provide strong circumstantial evidence that

PAHs are critical to the high incidence of neoplasia in salamanders from the lagoon.

TABLE 4. PAH ISOLATED FROM SLUDGE IN A SEWAGE EFFLUENT LAGOON CONTAINING A POPULATION OF TIGER SALAMANDER NEOTENES, *AMBYSTOMA TIGRINUM*, WITH A HIGH INCIDENCE OF NEOPLASIA (FROM ROSE, 1977)

Compound	Concentration (μg/kg, ppb)
Perylene	300.0
Pyrene	5.8
Fluoranthene	5.7
Alkyl pyrenes	4.9
Benz(a)anthracene	1.4
Chrysene	1.3
Triphenylene	0.5
Benzo(a)pyrene	0.5
Benzo(e)pyrene	0.2
Anthanthrene	0.2

A cancer-like hyperplasia was reported in the marine alga, *Porphyra tenera*, cultivated in coastal waters near industrial wastewater outfalls from the city of Ohmuta, Japan (Ishio *et al*., 1971; 1972a,b). The cancer could not be induced by exposing algae to diluted waste water. However, exposure of the leaves of the alga to bottom sediments from the outfall region for 80 to 320 min resulted in the production of cancerous growths within 36 days. The sediment was separated into several fractions and the greatest carcinogenic potency was found in the neutral fraction. Two carcinogenic compounds were isolated from this fraction. One was confirmed to be benzanthrone and the other which had the empirical formula $C_{25}H_{14}$ was tentatively identified as 12-hydrodibenz(cd, ghi)perylene.

Obviously, a great deal more research is required on identification of the causative agents of neoplasia outbreaks in aquatic organisms. This type of research requires close collaboration of histopathologists, cancer epidemiologists, and the environmental chemists.

Aquatic PAH and Human Cancer Risk

It has been estimated that from 50 to 90% of all human cancers are causatively related to environmental factors, mainly chemical carcinogens (Maugh, 1974; Wynder, 1976). The contribution of carcinogenic PAH in air, water, and food to human cancer is completely unknown. Near ubiquity of these compounds in the human environment indicates that PAHs could be important causative agents of human cancer.

There are many potential sources of PAH for humans, for example, drinking water, smoked, roasted or charcoal broiled foods, vegetables, vegetable fats and oils, and air pollutants (including cigarette smoke). It has been estimated that the amount of carcinogenic PAH consumed by man in drinking water is typically only about 0.1% of the amount accumulated from food (Andelman and Snodgrass, 1972). Drinking water from various sources typically contains 0.2 to 80 ng/ℓ (parts per trillion) BaP and 4 to 4,000 ng/ℓ total PAH (Table 5). Andelman and Suess (1970) made calculations based on four samples of drinking water and showed annual human consumption of PAH to be about 6, 9, 22, and 70 μg for the populations served by these water supplies. The 1970 World Health Organization Standards for Drinking Water (WHO, 1970) recommended that the concentration of total PAH in drinking water not exceed 0.2 μg/ℓ. Based on a daily human consumption of 2.5 ℓ, human consumption of drinking water with this concentration of PAH would result in the ingestion of 182.5 μg PAH per year. By comparison, smoke from 100 cigarettes (five packs) contains about 264 μg total PAH and 2.4 μg BaP (Severson et al., 1976).

TABLE 5. TYPICAL CONCENTRATION RANGES OF BENZO(a)PYRENE AND TOTAL PAH IN VARIOUS FRESH WATERS (CONCENTRATIONS ARE IN ng/ℓ)

Source	BaP	Total PAH	Reference
Groundwater (Germany)	0.4-7.0	10.9-123.5	Borneff & Kunte, 1969
Groundwater (Germany)	2-4	100-200	Hellmann, 1974
Well water (Germany)	2-15	100-750	Hellmann, 1974
Well water (England)	0.2-0.6	3.6-5.8	Lewis, 1975
Tap water (Germany)	0.5-9.0	29.2-125.5	Borneff & Kunte, 1969
Reservoirs (Moscow)	4-13	-	Il'nitskii & Rozhnova, 1970
Reservoirs (England)	0.7-3.8	9.1-43.2	Lewis, 1975
Rainwater (Koblenz)	4-80	200-4,000	Hellmann, 1974
Lake Constance (Germany)	0.2-11.5	25-234	Borneff & Kunte, 1964

Blumer (1972) suggested that petroleum pollution of the seas could pose a cancer risk to man through contamination of fisheries resources with carcinogenic PAHs and of recreational beaches with tar. Sullivan (1974) reviewed reports on BaP content of petroleum, rate of petroleum spillage in the sea, and contamination of marine organisms with PAH. He concluded that the amount of BaP in marine foods is higher (presumably due to oil pollution) than in non-marine foods and may pose a cancer hazard to human consumers of fishery products.

Available data indicate that river water may contain 0.0006 to 3.5 μg/ℓ (parts per billion) BaP and 0.02 to 3.8 μg/ℓ total PAH (Table 6). In most cases there is a direct relationship between PAH concentrations in river water and the degree of industrialization and other human activity along the banks and adjacent flood plain. Rivers remote from human activity are relatively uncontaminated.

TABLE 6. TYPICAL CONCENTRATION RANGES OF BENZO(a)PYRENE AND TOTAL PAH IN VARIOUS RIVER WATERS (CONCENTRATIONS ARE IN μg/ℓ)

Source	BaP	Total PAH	Reference
River Rhine at Mainz	0.05-0.11	0.73-1.50	Borneff & Kunte, 1964
River Rhine at Koblenz	0.01-0.06	0.5-3.00	Hellmann, 1974
River Aach at Stockach	0.004-0.043	1.44-3.10	Borneff & Kunte, 1965
Other German rivers	0.0006-0.01	0.20-1.00	Borneff & Kunte, 1964, 1965
Oyster River, CO USA	0.078-0.150	-	Keegan, 1971
Raskov region, USSR remote from human activity	10^{-5}-10^{-4}	-	Il'nitskii *et al*., 1971
Sunzha River below discharge of oil refinery	0.05-3.5	-	Samoilovich & Red'kin, 1968
Thames River, England	0.17-0.28	0.8-2.35	Acheson *et al*., 1976
Trent River, England	0.0053-0.504	0.025-3.79	Lewis, 1975
Severn River, England	0.0015-0.048	0.020-0.266	Lewis, 1975

Relatively little information is available concerning the concentrations of PAH in estuarine and oceanic waters. Apparently very few attempts have been made to precisely identify and quantify PAH concentrations in the oceans. Most of the data available are based on estimates of total aromatics by UV, IR, or fluorescence techniques--methods which may be subject to considerable interference from non-PAH material. Typical of this type of approach is the work of Zsolnay (1977) (Table 7). UV-absorbing "aromatics" represented 1 to 10% of the total hydrocarbons in the water samples. Aromatic hydrocarbon concentrations decreased with depth in most cases. Marty *et al*. (1978) measured the concentrations of aliphatic, alicyclic, and aromatic hydrocarbons in surface water, sea surface microlayer, and airborne particulates from the tropical Atlantic Ocean west of the Canary and Cape Verde Islands. Aromatic hydrocarbons represented 13 to 55% of the total hydrocarbons in the samples (Table 8). PAH in the water samples included phenanthrene, alkylphenanthrene, perylene, fluoranthene, and pyrene. Traces of benzofluoranthenes and benzopyrenes were also detected. The dominant PAH in the surface microlayer and airborne aerosols was phenanthrene (40% of the aromatic fraction). Mono-, di-, and tri-methylphenanthrenes were also abundant. No perylene, benzofluoranthenes, or benzopyrenes were detected. The authors hypothesized that the phenanthrenes were of biological origin and that the other PAHs were from industrial smoke and petroleum spillage. It would appear, therefore, that the concentrations of aromatics in marine surface waters are similar to those in ground water and well water.

TABLE 7. DISTRIBUTION OF HYDROCARBONS IN MARINE AND ESTUARINE WATERS; AROMATIC HYDROCARBON VALUES ARE RELATIVE AND ARE BASED UPON THE USE OF PHENANTHRENE STANDARD AT 254 nm (FROM ZSOLNAY, 1977)

Date Area	Depth (m)	Total hydrocarbons Mean & S.D. (μg/ℓ)	Aromatic hydrocarbons (based on UV abs.) (ng/ℓ)
1973			
Baltic	1	13.9±3.8	277±121
	10-50	5.4±1.8	52±9
	1 m above sediment	3.8±1.4	47±13
1973			
Nova Scotia	1	4.7±1.3	30.8±10
to Gulf Stream	10	3.5±1.8	16.8±4
	25	6.1±1.8	8.6±9
1974-1976			
Sargasso Sea	1	0.32±0.12	31±10
off Bermuda	30	0.47±0.25	1±0
	300	0.26±0.16	1±1
	1200	0.00	4
	2000	0.00	0
1975			
Mediterranean	1	16.0	148±36

TABLE 8. MEAN CONCENTRATIONS OF HYDROCARBONS INSURFACE SAMPLES OF SEAWATER, THE SEA SURFACE MICROLAYER, AND AEROSOLS COLLECTED 12 m ABOVE THE SEA SURFACE IN THE TROPICAL EASTERN ATLANTIC OCEAN (FROM MARTY et al., 1978)

Sample	Concentration of Total hydrocarbons	Composition of the hydrocarbon fraction	
		aliphatic & alicyclic hydrocarbons	aromatic hydrocarbons
Seawater	10 μg/ℓ	67%, 9% n-alkanes	33% (3.3 μg/ℓ)
Surface microlayer	39 μg/ℓ	45%, 6% n-alkanes	55% (21.4 μg/ℓ)
Aerosols	9 $\mu g/m^3$	87%, 2% n-alkanes	13% (1.2 $\mu g/m^3$)

Barbier et al. (1973) analyzed the hydrocarbons in surface water off the French coast at Brest. The concentration of total hydrocarbons was 137 μg/ℓ. Analysis of the hydrocarbon type distribution by UV spectrophotometry and mass spectrometry revealed that bicyclic aromatics represented 3.5% and PAH represented 2.5% of the total hydrocarbons present. Thus, the concentration of PAH in Brest seawater was approximately 3.4 μg/ℓ. Brown and Huffman (1976) analyzed a large number of water samples from the Atlantic, Mediterranean, and the Indian Ocean along the Persian Gulf oil tanker route. The mean concentration of nonvolatile hyhrocarbons in surface water from the Atlantic Ocean was 4 μg/ℓ with values from 1.3 to 13 μg/ℓ falling within one standard deviation of the mean. Mean relative aromatic concentration was 24% of the total nonvolatile hydrocarbons. Included in the total aromatic fraction were 1 to 4 ring aromatics plus sulfur-containing aromatics (benzo- and dibenzothiophenes). PAH represented less than half of the total aromatic fraction, so PAH concentrations in Atlantic Ocean surface water were about 0.4 μg/ℓ with a range of 0.13 to 1.3 μg/ℓ.

There are only a few reports dealing with concentration of specific PAHs in seawater. Seawater at Brest, France, was reported to contain traces of BaP (Saliot, 1969, as cited by Barbier et al., 1973). Armstrong et al., (1977) reported that a water sample taken approximately 15 m from an oil separator platform brine outfall in Trinity Bay, TX, contained 0.4, 0.2, and 0.6 μg/ℓ naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene, respectively (Table 9). No higher molecular weight PAHs were detected in the water despite the fact that the undiluted brine effluent contained significant concentrations of PAH in the dimethylnaphthalene-dimethylphenanthrene range.

TABLE 9. CONCENTRATIONS OF AROMATIC HYDROCARBONS IN THE BRINE EFFLUENT FROM AN OIL SEPARATOR PLATFORM IN TRINITY BAY, TX, AND IN THE WATER AND SEDIMENTS 15 m FROM THE BRINE OUTFALL (FROM ARMSTRONG *et al.*, 1977)

Compound	Brine effluent (μg/ℓ)	Water (μg/ℓ)	Sediment (μg/kg wet wt)
Benzene	3,300	1.50	-
Toluene	3,500	3.20	-
Xylene	2,400	-	-
C_2-Benzene	-	3.10	-
C_3-Benzene	650	0.80	600
C_4-Benzene	-	-	400
Naphthalene	300	0.40	200
1-Methylnaphthalene	370	0.20	500
2-Methylnaphthalene	500	0.60	500
Dimethylnaphthalenes	30	-	800
Trimethylnaphthalenes	260	-	10,000
C_4-Naphthalenes	-	-	400
Biphenyl	15	-	-
Methylbiphenyls	11	-	200
Dimethylbiphenyls	30	-	800
Fluorene	14	-	100
Methylfluorene	63	-	700
Dimethylfluorenes	42	-	900
Trimethylfluorenes	-	-	600
Phenanthrene	36	-	100
Methylphenanthrenes	63	-	600
Dimethylphenanthrenes	20	-	1,000
Trimethylphenanthrenes	-	-	500
Total aromatics	11,760	10.50	34,200

It can be concluded that fresh and marine waters, even from relatively polluted areas, contain enough low concentrations of PAH that they would not pose a carcinogenic hazard to recreational users of these water bodies.

More recent studies on BaP and total PAH contamination of marine organisms indicate that in the vast majority of cases these organisms contain low or undetectable levels of BaP and other PAH (Table 10). BaP concentrations in marine animals were usually in the 0 to 30 µg/kg range except in animals collected from severely polluted locations or from the immediate vicinity of creosoted pilings.

Cahnmann and Kuratsune (1957) measured the concentrations of eight PAH in the tissues of oysters, Crassostrea virginica, from the harbor of Norfolk, VA, an area fairly heavily polluted by domestic, industrial, and shipping wastes (Table 11). Total approximate PAH concentrations ranged from 700 to 1200 µg/kg wet weight. BaP represented only 0.2 to 0.3% of the total. Fazio (1971) measured the concentrations of nine PAH in oysters, C. virginica, from Galveston and Aransas Bays, TX. These oysters were much less contaminated than those from Norfolk, VA, despite the extensive oil refinery and port activity around Galveston Bay (Table 12). Interestingly, no BaP was detected in any of the oyster samples by a method demonstrated to be sensitive to 2 µg/kg wet weight or less. Samples from the most heavily contaminated station, an area in Galveston Bay closed to commercial oyster harvesting because of elevated sewage contamination, contained a mean total of 21.9 µg/kg wet weight of other PAHs. Oysters from uncontaminated regions of Galveston Bay and the relatively unpolluted Aransas Bay contained mean total PAH concentrations of 8.2 and 3.6 µg/kg, respectively. Lower molecular weight PAHs were more abundant than higher molecular weight PAH in both Galveston Bay and Norfolk, VA, oysters. In both cases fluoranthene and pyrene were the most abundant PAH in oyster tissues.

BaP concentrations as high as 1 to 5 mg/kg have been reported in some marine animals, and repeated consumption of such heavily contaminated animals would pose a cancer risk to humans. Heavily contaminated animals are usually unpalatable because of their oily smell and taste. The only report of BaP accumulation by a marine animal following an oil spill is that of Bories et al., (1976). Eleven days after a spill, Mytilus edulis, contained approximately 55 µg BaP/kg. In comparison, a charcoal broiled steak may contain up to 50 µg BaP/kg (Lijinsky, 1967), vegetable oils may contain up to 36 µg BaP/kg (Swallow, 1976), and smoked meats may contain up to 15 µg Bap/kg and 141 µg total BaP/kg (Panalaks, 1976). Typically food plants contain 0.08 to 30 µg BaP/kg (Carrier, 1977). Leafy vegetables and grains generally contain higher BaP concentrations than root vegetables. Thus, aquatic organisms do not ordinarily contain significantly higher concentrations of BaP and other PAHs than other human foods. However, consumption of aquatic organisms from regions severely contaminated with petroleum or industrial pollution should certainly be avoided.

In summary, the available evidence indicates that PAH-contaminated water and fishery products represent minor sources of PAH toxicity to man.

TABLE 10. SUMMARY OF ANALYSES OF THE CONCENTRATION OF BENZO(A)PYRENE IN THE TISSUES OF MARINE ANIMALS

Organisms	Location	BaP (µg/kg wet wt.)	Reference
Molluscs			
Mussels	Falmouth, Mass.		
(*Mytilus edulis*)	Little Sippewissett	<0.5	Pancirov & Brown, 1977
	Wild Harbor	0.5	Pancirov & Brown, 1977
	Oregon Bays	<0.1-30.2	Mix *et al*., 1977
	Vancouver Harbor, B.C.	<0.1-63	Dunn & Stich, 1975
M. edulis & *M. californeanus*	25 stations between Bodega Head - San Diego, Calif.	<0.1-8.2	Dunn & Young, 1976
M. californeanus	West coast Vancouver Island, B.C.	<0.1-0.2	Dunn & Stich, 1975
Oyster (*Crassostrea virginica*)	Long Island Sound	2	Pancirov & Brown, 1977
	Chincoteague, Va.	0.2	Pancirov & Brown, 1977
C. gigas	Tillmook Bay, Or.	<0.1	Mix *et al*., 1977
Gaper clam (*Tresus capax*)	Oregon Bays	<0.1-3.21	Mix *et al*., 1977
Softshell clam (*Mya arenaria*)	Oregon Bays	<0.1-6.66	Mix *et al*., 1977
Butter clam (*Saxidomas giganteus*)	Coos Bay, Or.	0.29-1.05	Mix *et al*., 1977
Clam (unidentified)	Chincoteague, Va.	0.3	Pancirov & Brown, 1977
Crustaceans			
Blue crab (*Callinectes sapidus*)	Chesapeake Bay	<5	Pancirov & Brown, 1977
Unidentified crab	Raritan Bay, N.J.	3	Pancirov & Brown, 1977
Shrimp (*Penaeus* sp.)	Palacios, Tx.	<1	Pancirov & Brown, 1977
Fish			
Cod (*Gadus* sp.)	Atlantic, 40 km off Toms River, N.J.	<1	Pancirov & Brown, 1977
Menhaden (*Brevoortia tyrannus*)	Raritan Bay, N.J.	1.5	Pancirov & Brown
Flounder (unidentified)	Long Branch, N.J.	<2	Pancirov & Brown, 1977

TABLE 11. CONCENTRATION OF SELECTED PAH IN THE TISSUES OF OYSTERS, CRASSOSTREA VIRGINICA, FROM THE HARBOR OF NORFOLK, VA (FROM CAHNMANN AND KURATSUNE, 1957)

Compound	Approximate concentration µg/kg wet weight
Fluoranthene	600 - 1000
Pyrene	100 - 160
Chrysene	20 - 40
Benz(a)anthracene	<10
Benzo(k)fluoranthene	8 - 12
Benzo(a)pyrene	2 - 6
Benzo(e)pyrene	<20
Benzo(ghi)perylene	1 - 5

TABLE 12. CONCENTRATIONS OF SELECTED PAH IN THE TISSUES OF OYSTERS, CRASSOSTREA VIRGINICA FROM THE TEXAS, GULF COAST (FROM FAZIO, 1981)

Compound	Concentration (µg/kg wet wt)					
	Station A		Station B		Station C	
	mean	range	mean	range	mean	range
Phenanthrene	-		-		2.2	0.0-6.9
Fluoranthene	1.7	1.7	3.0	1.6-5.6	7.8	2.8-14
Pyrene	0.9	0.8-1.0	1.8	1.3-5.9	6.5	3.8-13
Chrysene	0.5	0.3-0.6	0.2	0.0-1.7	0.6	0.0-2.0
Benzo(b)fluoranthene	0.3	0.0-0.5	1.2	0.5-1.8	2.2	0.0-4.1
Benzo(k)fluoranthene	-		0.1	0.0-0.6	0.4	0.0-1.1
Benzo(a)pyrene	-		-		-	
Benzo(e)pyrene	0.2	0.0-0.4	1.2	0.0-4.0	2.1	0.8-4.2
Perylene	-		0.7	0.0-3.5	0.1	0.0-1.5

Station A, Aransas Bay oyster reefs relatively uncontaminated from domestic and petroleum operations; Station B, Galveston Bay oyster reefs approved for commercial harvesting; Station C, Galveston Bay oyster reefs closed to commercial harvesting due to domestic sewage contamination.

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